An Investigation of

Some Biochemical and Cellular Properties of

Subretinal Fluids

by

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SUMMARY

1) Subretinal fluids were studied biochemically and morphologically to further on understanding of the relationship between the chemical and cellular constituents of the fluids and the development of retinal detachments and proliferative vitreoretinopathy (PVR).

2) Subretinal fluid (SRF) specimens were obtained internally through the retinal break during the initial stage of vitrectomy.

3) Total protein contents of forty six SRF samples were assayed using the Lowry's method. It was found that the larger the size of retinal break, the lower the total protein level. In addition, the protein contents of subretinal fluids increased with the duration of retinal detachment. However, there was no relationship between the total protein content in SRF and the severity of PVR. These results could, in part, be explained by the fluid dynamics of retinal detachment.

4) Basic fibroblast growth factor (bFGF) contents were quantitated in 43

samples of SRF. The data showed that as the severity of PVR increased, the levels of bFGF from these SRF samples likewise increased. Levels of bFGF in the SRF of eyes with previous cryotherapy were found to be higher than those without previous cryotherapy. The elevated levels of bFGF might play a role during the course of development of PVR.

5) Four types of cells were found in SRF samples: neuron retinal cells, heavily pigmented cells, retinal pigment epithelium and macrophages. As judged from data obtained from the present study, RPE cells may be a major component of proliferative subretinal and epiretinal membranes. Iris or ciliary pigmented epithelial cells and macrophages could also contribute to the proliferative processes.

6) In the SRF samples of eyes with prior cryotherapy, there were a large number of RPE cells, neuron retinal cells and dispersive lipofuscins which might suggest many RPE cells were disintegrated. Findings obtained from the present study may provide further evidence to the pathogenetic mechanism(s) of the development of PVR.

CONTENTS

CHAPTER 1

INTRODUCTION

Retinal detachment (RD) is a separation of the neuro-sensory retina from the retinal pigment epithelium (RPE) by the subretinal fluid (SRF). Surgery to repair rhegmatogenous retinal detachment is successful in more than 90% of cases, and the most common cause of failure is progressive vitroretinal traction due to proliferative vitreoretinopathy (PVR) (1). The formation and gradual contraction of fibrocellular epiretinal membranes result in a marked distortion of the retinal architecture and produce complex retinal detachments.

It has been shown that PVR is a RPE - mediated disease (2-4), but the mechanisms of PVR have not been well established and the biological mediators involved in this intraocular proliferative process have yet to be determined.

Basic and acidic fibroblast growth factors (FGF) are two closely related peptides (5) that act as potent mitogens and differentiation factors for mesoderm and neuroectoderm derived cells, including RPE cells (5,6).

It has been demonstrated that acidic fibroblast growth factor (aFGF) is 30-

100 fold less potent than that of basic fibroblast growth factor (bFGF) (7,8). Basic and acidic FGF contribute 8% and 0.15% of the total mitogenic activity present in crude retinal extract, respectively (9).

Schweigerer et al (10) showed in 1987 that bFGF was synthesized in RPE cultured cells and localized (11,12) in the neuroretina at various cellular and extracellular locations including the interphotoreceptor matrix occupying the extracellular space between the neuroretina and the RPE (13).

Using an anti-aFGF antiserum, Baudouin (14) reported the presence of large amount of this growth factor in the cytoplasm of pigment and nonpigment epithelia cells of pars plana and ciliary processes. It has been suggested that the presence of aFGF in these locations might play a role in the pathogenesis of anterior PVR (15,16).

In 1990, Leschey et al (6) demonstrated that DNA synthesis in RPE cell could be stimulated by FGF. The enhanced DNA synthesis resulted in an increase in the RPE cell number after incubation *in vitro* (6).

An immunohistological study has been conducted recently by Baudouin et al (17) using intravitreal and subretinal fluid cells obtained from patients suffering from PVR in various stages of development, and acidic FGF was identified in all vitreous and subretinal specimens.

There has, so far, been no report on the analysis of basic FGF in subretinal fluid. If the release of bFGF provides an important stimulus for PVR in eye disorders, the presence of bFGF should, in theory, be detected in subretinal fluids.

Cellular studies in PVR have so far been performed mostly in preretinal membranes and tissues. Investigations of the cellular contents of the subretinal fluids have been relatively few.

Five major cellular constituents were identified in epiretinal membranes removed during vitrectomy, namely RPE cells, macrophage, fibroblast-like cells, fibrous astrocytes, and myofibroblastic-like cells (18). However, the origins of these cells remain controversial. By means of immunohistochemical techniques, Hiscorts et al (19) suggested that the epithelial cells in the epiretinal membranes were probably derived from the retinal pigment epithelium.

In 1989, Jerdan et al reported (20) using immunocytology that the RPE cells in PVR membranes occurred both as single cells and as large clusters and macrophage tended to occur as single cell.

Other studies had also indicated that the RPE cells were the major cellular components of epiretinal membranes and the ultrastructural characteristics of these membranes had been well documented (21-23).

Few studies have so far been conducted on the cellular components of subretinal fluid surrounding the detached retina and the proliferative tissues. However, Baudouin showed (24) the presence of heavily pigmented cells, poorly pigmented cells, large totally unpigmented macrophage-resembling cells, smaller unpigmented cells, and lymphocytes in subretinal fluids, but none of the cells were positive for anti-macrophage antibodies in subretinal fluids.

The cellular components in subretinal fluids in RD or PVR have not been thoroughly studied. The presence of different types of cells in SRF could yield informative data in elucidating the biological mechanisms leading to RD and PVR.

The total protein contents in subretinal fluids had been studied previously (25-28). Nevertheless, conflicting results were reported in literature. Some investigators (29,30) had shown that the total protein concentration in subretinal fluids increased with increasing duration of RD, while other reports (28,31) were not able to confirm this correlation.

In most of the previous mentioned studies, subretinal fluid samples were collected extraocularly during internal release of SRF by choroidal perforation. These SRF samples were invariably contaminated with microscopic choroidal hemorrhage during perforation. This might yield erroneous results.

The primary aims of this study of subretinal fluids in RD include:

1) to obtain subretinal fluids internally through the retinal breaks at the beginning of vitrectomy, thus avoiding the contamination;

2) to determine the relationships between the total protein content in SRF and the size of the retinal break, the duration of RD and, the severity of PVR;

3) to measure quantitatively the level of basic FGF, and to determine the relationship between the level of bFGF and the severity of PVR; and

4) to analyze the cellular components in SRF in an attempt to identify the origins of the cellular elements contributing to the retinal detachment.

CHAPTER 2

LITERATURE REVIEW

2.1 Anatomy of retina and vitreous

The retina covers the aspect of the posterior two - thirds of the wall of the eye globe. As a matter of fact, the retina is a multilayered sheet of neural tissue closely applied to a single layer of pigment epithelial cells, which in turn is attached to the Bruch's membrane (32).

The invagination of the lateral optic vesicles during embryonic development forms the double - walled secondary optic vesicles. The inner wall forms light - sensitive sensory retina. The outer wall thins to a single layer, the retinal pigment epithelium. The central space of the vesicles collapses into a potential space (32).

The sensory retina may be divided into a) a central portion (macula), which contains the fovea centralis that functions in photopic vision, and b) four peripheral quadrants that function in spatial orientation and in reduced light (scotopic vision). The central retina - the macula, located between, extends temporally from the optic

disk to about 2 disk diameters lateral to the fovea centralis. Fovea centralis functions in bright illumination (photopic vision), form vision and color vision. Rod photoreceptor are most common in the peripheral quadrants and function in dim illumination (scotopic vision) (33).

The retinal pigment epithelium is a monolayer of specialized cells adjacent to the receptor outer segments of the retina and extending from the optic nerve to the ora serrata. The cells of the pigment epithelium normally have a hexagonal shape and structural polarity. The external (basal) surfaces are intimately associated with the retinal photoreceptor. The pigment epithelial cells contain pigment granules (lipofuscient and melanin) that are mainly located near the apical part of the cell. Neighboring pigment epithelial cells are joined by specialized ultrastructural attachments between the adjacent cell membranes (33).

These areas of attachment seal the lateral intercellular space from the subretinal space contributing to the barrier that prevents the free exchange of macromolecules between the photoreceptor and the choroidal circulation. Thus, molecular movement is mainly through the pigment epithelial cells and therefore, is regulated by the metabolic pathways of the cells (34).

The vitreous consists of about 99% water. The remaining 1% includes cellular components, collagen and hyaluronic acid, which give it its specific physical character. The vitreous is a clear, avascular, gelatinous body that comprises

two-thirds of the volume and weight of the eye. It fills the space bounded by the lens, retina, and optic disk. Since it is quite inelastic and impervious to cells and debris it plays an important role in maintaining the transparency and form of the eye (35).

2.2 Rhegmatogenous retinal detachment

2.2.1 Retinal breaks

Retinal breaks are either tears or atrophic holes. The fovea and the peripheral retina are the sites of predilection for retinal breaks. In both places, the internal limiting membrane and retinal parenchyma are thin. In retina tears, the retina is torn by mechanical force, with the formation of a distinctive feature of an operculum or avulsed tissue. The size of the break may vary tremendously (36).

2.2.2 Retinal detachment and subretinal fluid

Because the retina is only loosely adherent to the pigment epithelium by interdigitating villi, the two layers separate easily allowing fluid (subretinal fluid) to fill up the subretinal space. The production of rhegmatogenous retinal detachment is dependent upon a number of factors, i.e. a retinal break; a force sufficient to

break the connection between the neurosensory retina and the RPE, and transfer of fluid from the vitreous cavity to the space behind the retina (37).

2.3 Proliferative vitreoretinopathy

Surgery to repair rhegmatogenous retinal detachment is successful in more than 90% of cases, and the most common cause of failure is progressive vitreoretinal traction due to PVR (1). The formation and gradual contraction of fibrocellular epiretinal membranes result in a marked distortion of the retinal architecture and produce complex retinal detachments. The classification of PVR (Table 2.1) is structured to grade the severity of the process. It also reflects a continuous pathologic spectrum of changes, ranging from mild evidence of intravitreal proliferation to severe structural damage complicating retinal detachment and indicating the extent of epiretinal membrane formation (1).

Table 2.1 Classification of retinal detachment with proliferative vitreoretinopathy (1)

Grade	Name	Clinical Signs
A	Minimal	Vitreous haze, vitreous pigment clumps
B	Moderate	Winking of the inner retinal surface,rolled edge of retinal break, retinal stiffness vessel tortuosity
C	Marked	Full thickness fixed retinal folds
C1		One quadrant
C2		Two quadrants
C3		Three quadrants
D	Massive	Fixed retinal folds in four quadrants
D1		Wide funnel shape
D2		Narrow funnel shape
D3		Closed funnel

2.4 Total protein in subretinal fluid

Many researchers studied the protein concentration of the subretinal fluid and much attention had been focussed to the inter-relationship between the duration of the RD and the level of protein in the subretinal fluid. Some authors showed the increase of protein content in SRF with increasing duration of detachment (29,30), while other studies were unable to confirm this (28,31).

Two reports noted that subretinal fluid protein content was higher in more extensive detachments than in smaller ones (30,37). Since old detachments tend to be more extensive, it is not clear what factor is contributory to the subretinal protein level. However, investigation carried out by Sweeney et al (37) reported an inverse relationship between the protein concentration in subretinal fluid and the size of retinal break, i.e. the smaller the break, the higher the protein content.

The total protein content of plasma (70 mg/ml) were reported to be about 150 times higher than that of vitreous (0.5 mg/ml), while the protein contents of subretinal fluid falling in between plasma and vitreous (26,27,38). Since the concentration of protein in the vitreous is much lower than that in the subretinal fluid or in the plasma, it is unlikely that the retina could contribute much to the large volume of fluid that often accumulated in the subretinal space. It is generally thought that the protein in subretinal fluid is derived largely from plasma transudation (26,27,39).

2.5 Fibroblast growth factor

There are seven members of the FGF gene family: 1) basic FGF (40), 2) acidic FGF (41), 3) int-2 protooncogenes (42), 4) hst protooncogenes (43), 5) FGF-5 (44), 6) FGF-6 (45), and 7) keratinocyte growth factor (KGF) (46).

Basic and acidic FGF are two closely related peptides of relative molecular mass (Mr) = 16,000 that act as potent mitogen and differentiation factors for a wide variety of mesoderm and neuroectoderm derived cells. Basic FGF (bFGF, pI = 9.6) was first identified by its ability to cause the proliferation and phenotypic transformation of BALB C 3T3 fibroblasts (47), whereas acidic FGF (aFGF, pI = 5.6) was first identified by its ability to cause the proliferation and delayed differentiation of myoblast (48). It was subsequently demonstrated that both aFGF and bFGF are able to stimulate endothelial cell proliferation (49).

Both aFGF and bFGF have been purified to homogeneity and their amino acid sequences have been determined (50). It is now clear that growth factors isolated from eye, brain, etc. are structurally and biologically identical and they are very similar to bFGF or aFGF. The availability of pure aFGF and bFGF has led to the recognition of a wide spectrum of activities for these two mitogens, most notably their potential to act as angiogenic factors *in vivo*. Basic FGF and aFGF are multifunctional, i.e. they can either stimulate proliferation and induce or delay

differentiations (51).

2.5.1 Structure of basic and acidic FGF and their gene

Basic FGF has been purified from most mesoderm- or neuron-ectoderm-derived tissues or cells that have in common a strong angiogenic potential (9,52). Structural studies have shown that bFGF is a single chain peptide composed of 146 amino acids, which can also exist in an NH_2-terminally truncated form with the first 6 amino acids missing (9).

Basic FGF and aFGF possess two potential binding domains for heparin. Both domains are believed to be involved in the strong affinity of FGF toward heparin (50). The high degree of homology between aFGF and bFGF suggests that they are derived from a single ancestral gene. The FGF genes have been cloned and complementary DNA sequences of both bFGF and aFGF have been synthesized (40). In human cells, the bFGF gene is localized on chromosome 4 whereas the aFGF gene is localized on chromosome 5 (53). It seems that through a process of gene duplication and evolutionary divergent, bFGF and aFGF have become separate gene products.

2.5.2 Expression of bFGF and aFGF in neuroretina and pigmented epithelial cells

Basic and acidic FGF had been purified from retinal tissue (9,52). It has been shown that aFGF is 30-100 fold less potent than that of bFGF. The contribution of aFGF to the total mitogenic activity is hence significantly less than that of bFGF. Basic and acidic FGF contribute 8% and 0.15% of the total mitogenic activity present in crude retinal extract, respectively (9). In a study carried out in 1987, it was found that bFGF was synthesized in culture retinal pigment epithelial cells (18).

Using an anti-acidic fibroblast growth factor antiserum, Baudouin demonstrated the presence of great amounts of this growth factor within the cytoplasm of pigment and nonpigment epithelial cells of the pars plana and ciliary processes (14).

Recent evidence obtained from Western blot analysis as well as light and electron microscopy indicates that bFGF has been found both in the cytosolic and nuclear fractions of human retinal pigment epithelium (19). The bFGF had also been shown (11,12) to be in the neuroretina to various cellular and extracellular locations, including the interphotoreceptor matrix that occupies the extracellular space between the neural retina and RPE (13).

2.5.3 The FGF receptors

All cell types (including RPE cell) that respond to bFGF or aFGF bear specific FGF cell surface receptors and several high affinity FGF receptors (FGF-R) have recently been cloned and these exists at least four families of FGF receptors (9,52). Scatchard analysis of radio-labelled FGF indicated that dystrophic RPE possess only 29% the number of surface receptors as compared to congenic normal cells (54). This suggests the importance of trophic factors in the normal functioning of the retina.

2.5.4 *In vitro* biological effect of FGF

Basic FGF is a potent mitogen for mesoderm-derived cells in triggering cell proliferation. Basic FGF is mitogenic both for cells seeded at clonal density and low density cultures. The addition of bFGF greatly reduces their average cellular doubling time (55). This is primarily due to be the shortening of the G1 phase of the cell cycle (56).

Recent study revealed that DNA synthesis in RPE cell is stimulated by acidic and basic FGF. The effects of aFGF and the bFGF have been shown to be dramatic, whether used alone or in combination (6). The enhanced DNA synthesis

results in an increase in RPE cell number after incubation *in vitro* (6).

2.5.5 FGF in retinal diseases

The retinal pigment epithelium (RPE) form a monolayer of cells beneath the sensory retina which is normally mitotically inactive. However, several types of insults may cause initiation of RPE proliferation, manifested clinically by hyperpigmented retinal scars (56). Proliferation of RPE cells also occur after retinal detachment and the proliferation of RPE cells has been implicated in the pathogenesis of PVR (57). With the exception of the capillary endothelial cells, the RPE cells express the basic fibroblast growth factor (bFGF) gene and they contain transcripts of the bFGF gene which are translatable into bioactive bFGF (58).

Fibroblast growth factor production had been recently emphasized in anterior proliferative vitreoretinopathy which is characterized by cellular proliferation in the area of the vitreous base and traction on the anterior retina (15,16).

Ten samples of vitreous obtained from patients with retinal detachment were used for the measurement of basic FGF in 1990 (59), and bFGF was demonstrated in two of four vitreous samples in eye with severe PVR (Stage D). The other six vitreous were from patients with the retinal detachment underwent vitrectomy for a number of conditions, including retinal detachment resulting from the following:

macular hole, giant tear and expulsive choroidal hemorrhage as well as rhegmatogious retinal detachment with stage C1 PVR. In all of these latter mentioned patients, the level of bFGF was undetectable.

Acidic FGF has also been shown in intravitreal and subretinal fluid cells obtained from patients suffering from PVR of various stages of the disease development (17).

Diabetic retinopathy which could also induce tractional retinal detachment is a disease of the retinal microvasculature that reflects a compromise of metabolic, endocrine, and hematologic systems but also results from unique local conditions.

FGF is a potent mitogen for cultures of retinal derived vascular endothelial cells. The production of bFGF by bovine capillary endothelial cells in culture suggests a role of FGF in the regulation of neovascularization. FGF has been localized in the basement membrane of capillaries where it is presumed to be bound in an inactive state to heparin (60). This observation raises an interesting possibility that, following an injury to the capillaries, latent FGF may release from the basement membrane and rapidly stimulates the initiation of neovascularization by its action in endothelial cells, pericyte and fibroblasts. Results obtained from clinical studies (59) have also suggested the involvement of FGF in retinal neovascularization. Levels of bFGF have recently been reported in the vitreous of 36 patients undergone vitrectomy for a variety of retinal conditions including

proliferative diabetic retinopathy. Approximately half of the 17 patients with proliferative retinopathy had elevated (>30 ng/ml) levels of bFGF in the vitreous, and six of the eight patients with active proliferative retinopathy had elevated FGF levels as compared to only two of seven with inactive disease. The elevation in the levels of bFGF is thought to be sufficient in stimulating the DNA synthesis of endothelial cells *in vitro*. It appears that FGF plays a key role in neovescular proliferation.

2.6 Cellular study of proliferative vitreoretinopathy

2.6.1 Experimental study

Research in the area of intravitreal cell proliferation began in the late 1960s. Machemer et al (61) designed an experimental model of retinal detachment in the owl monkey which allowed for the longitudinal study of persistently detached retinas over long period of time. This model was able to reproduce the constellation of proliferative signs, including the migration of pigmented cells into the subretinal space and into the vitreous cavity. These pigmented cells showed considerably phagocytic and mitotic activity. A gradual loss of pigmentation in these cells was noted after multiple cell divisions. It was not clear, however, whether the origin of these cells was from macrophage or from the retinal pigment epithelium. These pigmented cells have previously been referred to as "pigment epithelial

macrophage". Subsequent investigations (21,62) showed that these cells were able to form a monolayer on the retinal surface and they were histologically and ultrastructurally indistinguishable from normal pigment epithelium. After from the presence of pigmented cells, it was noted that fibrocyte-like cells in the form of membrane-like structures with nearly collagen accumulations (21). These fibroblast - like cells were found both under and in front of the retina, along vitreous surfaces, and behind the lens; the term "massive periretinal proliferation (MPP)" was therefore introduced (21).

To provide a reproducible animal model for intravitreal cellular proliferation, an study had been undertaken by Algvere and Kock (63,64). This was done by injecting cells into the vitreous cavity, and a variety of cell types of autologous, homologous, or heterologous origin was used. Algvere and associates found a distinct growth of injected cells which formed strands within the vitreous. Most eyes showed retinal detachment, and some exhibited traction of optic nerve tissue into the vitreous. Cellular inoculations used by other researchers (65-72) included RPE cells, glial cells, retinal Muller's cells, fibroblasts, chondrocyte, red blood cells and macrophages. They all produced substantial PVR-like pathologic changes.

2.6.2 Pathogenesis of intravitreal cell proliferation

It has been shown that viable RPE cells are released into the vitreous cavity at the time of retinal tear formation (21). Some RPE cells may probably remain attached to the retinal flap after the retina was torn. Subsequently, these cells on the flap migrate into the vitreous, possibly caused by the presence of chemo-attractant(s) in the vitreous.

Some research workers observed, however, the dispersion of a cloud of pigment at the time of trans scleral cryotherapy (73). The intravitreal dispersion of viable RPE cells after cryotherapy has been shown experimentally as well as in clinical situations (74). Cryotherapy causes the breakdown of the blood-ocular barriers with subsequent influence of serum- derived proliferative factor(s) and chemo-attractant(s) which may, in turn, trigger the proliferation of RPE cells. Some serum factors that stimulate RPE cell proliferation and migration have been identified e.g. fibronectin (FN) and platelet-derived growth factor (PDGF) (75,76).

2.6.3 Cellular components of proliferative tissue

Five major cellular constituents have been identified in epiretinal membranes removed during vitrectomy: retinal pigment epithelial cells, macrophage, fibroblast-

like cells, fibrous astrocytes, and myofibroblastic-like cells. The origins of these cells are controversial (18). An examination of membranes produced during retinal detachment in the owl monkey model demonstrated that astrocytes (glial cells) were originated from small dehiscence in the internal limiting membrane of the retina (77). Glial cells were also present in membranes removed from human eyes with retinal detachment and PVR (78).

Animal experiments by Machemer and Laqua et al (21) reported the presence of that macrophage, fibroblast-like cells, and pigment epithelial cells in eyes with PVR, and it was suggested by these workers that retinal pigment epithelial cells and the fibroblast-like cells played a role in the preretinal membrane formation. An analysis of specimens obtained from human eyes with PVR and retinal detachment confirmed this observation (79). Two experiments suggested that retinal pigment epithelial cells transformed into macrophage and fibroblast-like cells (80,81). Other study, however, conducted that fibrocyte-like cells may not be derived from the differentiation of retinal pigment epithelial cells (82). Other possible origins included transformation of glial cells, hyolocytes, vascular perithelial cells (83), or mesenchymal cells that originated within the choroid or optic nerve (82).

Macrophage found in tissue specimens from eyes with PVR may actually include three cell types: wandering tissue macrophage, macrophage derived from retinal pigment epithelial cells, and blood stream macrophage (21). Myofibroblastic-like cells have numerous microfilaments that probably are actin. Several cell types

may be able to develop myofibroblast-like qualities (83). The cellular elements in PVR are found in a collagenous matrix. Each cell type is capable of synthesizing collagen, which gives a whitish appearance to membranes. Clear preretinal membranes lack collagen and are composed of just cellular elements. Collagen fibrils, however, strengthen and stabilize the membrane (84).

2.6.4 Cellular components of subretinal fluid

Few studies have so far been conducted on the cellular components of the subretinal fluid surrounding the detached retina and proliferative tissues. In 1975, Feeney (85) observed that whole cells found in most specimens included erythrocytes and macrophage. Intact pigment epithelial cells were identified by their original shape and polarity. Other cell types were rather difficult to classify with any degree of certainty.

Using cytologic and immunocytologic procedures, 30 specimens of vitreous or subretinal fluid removed from patients with PVR have recently been studied. Five main types of cells: heavily pigmented cells, poorly pigmented cells, large totally unpigmented macrophage-resembling cells, smaller unpigmented cells, and lymphocytes. B-cells have been identified. However, no T-lymphocytes could be identified. In addition, no cell was positive for anti - macrophage antibodies, and macrophage-specific staining was negative in all cells (24).

CHAPTER 3

MATERIALS AND METHODS

3.1 Specimens

Patients undergoing pars plana with 3-port vitrectomy were included in this study. All eyes had rhegmatogenous retinal detachment with various degree of PVR requiring vitrectomy. The subretinal fluid was drawn internally using a blunt tip needle on a 1-ml syringe passed through the retinal break during the initial stage of vitrectomy before intraocular infusion was begun. In rare cases, when retinal break was not detectable during operation, vitrectomy produced for SRF collection and fluid-gas exchange.

3.2 Determination of total protein

3.2.1 Study population

This study was performed on 46 specimens of subretinal fluid surgically obtained from 46 patients. Patients age ranged from 11 to 82 years, with 16 women and 30 men suffering from retinal detachment with grade A to D degrees of PVR. The severity of PVR was graded in a masked fashion using the system proposed by the Retinal Society Terminology Committee (1).

The sera from 12 patients and vitreous from 11 fresh autopsy cases without any known ocular disease were also collected and used as controls. All subretinal fluid samples, vitreous and serum specimens were stored frozen at -75 °C until use. Repeated freeze and thaw cycles were avoided.

3.2.2 Quantitation of total protein

Subretinal fluid, sera and vitreous were diluted 50 - 100 folds, 1000 folds, and 2 folds, respectively. The protein contents of all the specimens collected in this study was determined by the method according to Lowry et al (86). Crystalline bovine serum albumin (Fractron V power, A 4503, Sigma Chemical Company, St. Louis, Missouri, USA) was used as a standard.

Materials:

1. Complex-forming reagent: Immediately before the determination of the protein content of each sample, a

complex - forming reagent was made by mixing the stock solutions A, B, and C in the proportion 100:1:1 (vol : vol: vol), respectively, and the composition of solution A, B and C are detailed as below:

Solution A: 2% (w/v) Na_2CO_3 in distilled water

Solution B: 1% (w/v) $CuSO_4.5H_2O$ in distilled water

Solution C: 2% (w/v) Sodium potassium tartrate in distilled water

2. 2N NaOH.

3. Folin Reagent (Merch, Darsmstadt, Germany): 1N Concentration was prepared.

4. Standards: A stock solution of the protein standard containing 0.1 mg/ml protein in distilled water was prepared. 2 ml aliquots of the protein standard were frozen at 20 °C until use. Standards were prepared by diluting the stock solutions with distilled water as follows:

Protein Conc. (ug/ml)	0	10	20	30	40	50	60	70	80	100
Stock Solution (ul)	0	20	40	60	80	100	120	140	160	200
Water(ul)	200	180	160	140	120	100	80	60	40	0

Procedures:

1. 0.1 ml of 2 N NaOH was added to 0.1 ml diluted sample or standard. The sample or standard was allowed to hydrolyse at 100 °C for 10 minutes using a boiling water bath.
2. 1 ml of freshly prepared complex - forming reagent was then added to the hydrolysed mixture after the hydrolyzate had been cooled to the room temperature. The resulted solution was allowed to stand at room temperature for 10 minutes.
3. 0.05 ml of Folin reagent was added to the solution resulted in step 2, and mixed with a vortex mixer. The mixture was allowed to stand at room temperature for 30 minutes
4. The absorbance of each sample or standard was read at 750 nm using a "Gilford Response" UV / VIS spectrophotometer (Gilfod Instruments Laboratories, Inc., Oberlin, Ohio, USA) fitted with an autosampling device.
5. A standard curve was prepared by plotting the absorbance against the concentrations of protein standards and the standard curve constructed was used to determine the concentrations of proteins in specimens collected in this study.

3.3 Determination of bFGF

3.3.1 Study population

Subretinal fluid specimens were obtained during retinal detachment surgery from 43 patients (16 women and 27 men, age range 18-82, mean age 47 years old) suffering from retinal detachment with various degrees of PVR: 7 patients of below grade C1, 9 of mild PVR (grade C1-C3) and 27 of severe PVR (grade D1-D3).

The patients with PVR grade more than C1 were divided into two groups. The first group consisted of 16 patients who had been treated previously by cryotherapy but failed. The duration between the two operations ranged from 2-52 weeks. The second group consisted of 11 patients, who had not been treated by cryotherapy previously. Nine patients who had other eye operations previously, such as cataract surgery, vitrectomy etc. were also studied, but not included in statistical analysis.

Sera from 12 patients and vitreous from 10 fresh autopsy cases were collected and used as controls.

Sterile technique was maintained throughout the study. Samples for measuring the level of bFGF were stored frozen at -70 °C, and repeated freeze and thaw were avoided.

3.3.2 Quantitation of bFGF

Levels of bFGF in subretinal fluids, vitreous and serum samples were determined using Quantikine HS (high sensitivity) immunoassay kits produced by R & D Systems, Minneapolis, Minnesota, USA.

The assay was a "sandwich" enzyme immunoassay employing a monoclonal antibody specific to bFGF coated onto microtiter plate for trapping all basic FGF in both the samples and the standards. 0.2 ml of standard or sample was applied to each well of the microtiter plate. The plate was then sealed with adhesive strip and incubated for 2 hours at room temperature. At the end of the 2 hours incubation period, both the standards and samples were aspirated. All 96 wells of the microtiter plate were then washed three times with wash buffer provided, e.g. 300 ul of wash buffer per well, this was dispensed using an 8 channels auto-pipette. After washing, a horseradish peroxidase-linked polyclonal antibody specific to bFGF (e.g. 200 ul per well) was used for quantitative measurement of the immobilized bFGF in each well. This was followed by two hours incubation at room temperature, and the plate was sealed with adhesive strip as described previously. After removing any unbound antibody-enzyme conjugate at the end of the 2 hours incubation period, a substrate solution (200 ul) containing hydrogen peroxide and tetraethyl benzidine was added to each well of the microtiter plate for color development. The color produced was proportional to the amount of bFGF bound in the initial capturing step. After 30 minutes, the color development was stopped by the addition of 50 ul of 2 N sulfuric

acid to each well and the intensity of the color in each sample was measured using a Dynatech MR 600 ELISA plate reader at 450 nm.

A standard curve ranging from 10 pg/ml to 640 pg/ml was prepared using purified recombinant human bFGF provided by the manufacturer. This was done by plotting the optical densities of the standards against the concentrations of bFGF in the standard wells of the microtiter plate. By comparing the optical density of the samples to the standard curve, the concentrations of bFGF in the unknown sample was then determined. All samples and standards were assayed in duplicates.

3.4 Cellular studies

3.4.1 Study population

This study was performed on 35 specimens, suffering from retinal detachment with proliferative vitreoretinopathy. The duration of retinal detachments of these patients ranged from 0.5 to 12 months.

3.4.2 Fixation of samples

Standard glass microscope slides were pretreated with organosilane. An 80

ul fresh subretinal fluid samples was drawn up using a clean pipette and was placed in the receptacle well which was attached to the microscope slide and filter paper component (Fig.3.1) The sample was spun at 600 RPM for 5 minutes in a Shandon Cytospin 2 centrifuge (Sewickley, PA) and allowed to air dry. The slides were then fixed for 10 minutes in 95% ethanol at room temperature. Ten slides were prepared from each specimen.

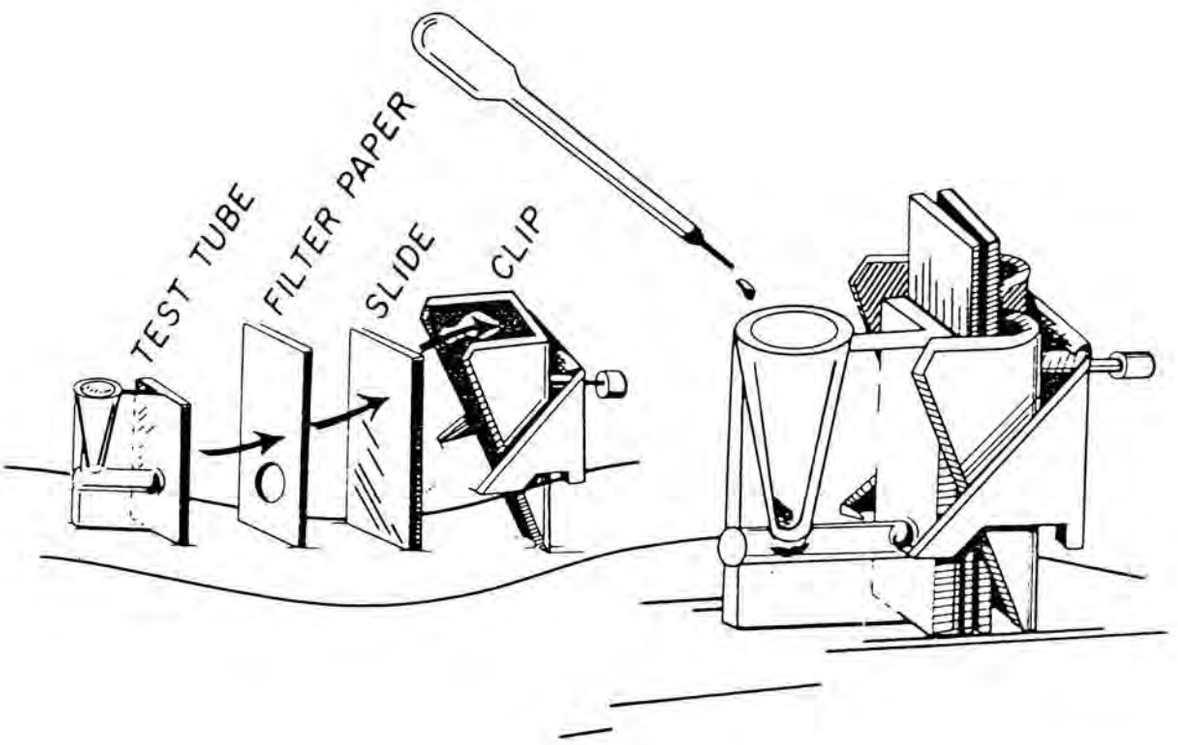

Fig. 3.1 Cytocentrifugation Components (From Chess, J.) (87)

3.4.3 Immunocytochemistry

Primary antibodies used in studying the origin(s) of cells present in subretinal fluid.

Antibodies	Source	Dilution	Specificity
Anti-Human Neuron-Specific Enolase (NSE)	DAKO Japan Co. Ltd, Kyoto, Japan.	1:100	Mouse monoclonal antibody, reacts with neurons from human and with APUD cells, and myelinated and unmyelinated nerve fibers in human tissue specimens.
Anti-Human Myeloid/Histiocyte Antigen (DAKO-MAC387)	DAKO Japan Co. Ltd, Kyoto, Japan.	1:100	Mouse monoclonal antibody, reacts with macrophages. In blood smears from peripheral blood a positive staining of monocytes.
Anti-Human T Cell, CD45RO (DAKO-CD45RO, UCHL1)	DAKO Japan Co. Ltd, Kyoto, Japan.	1:100	Mouse monoclonal antibody, labels most thymocyte, a subpopulation of resting T cells within both the CD4 and CD8 subsets, and mature activated T cells.
Anti-Human B Cell, CD20 (DAKO-CD20, L26)	DAKO Japan Co Ltd, Kyoto, Japan	1:50	Mouse monoclonal antibody, reacts with the majority of B cells present in peripheral blood and lymphoid tissue.

Secondary antibodies:

Biotinylated rabbit anti-mouse immunoglobulins were purchased from DAKO, Japan Co. Ltd, Kyoto, Japan.

Peroxidase-conjugated streptavidin:

Peroxidase-conjugated streptavidin were purchased from DAKO Co. Ltd, Japan.

Immunological procedures:

1. Slides prepared after samples had been fixed were gently rinsed with Tris - buffered saline - 20 mM Tris / HCl, 150 mM NaCl, pH 7.6 (TBS) and then bathed in TBS for 5 minutes.
2. Excess fluid around the cells of each slide was removed.
3. Non - specific binding sites on cells of each slide were then blocked using normal rabbit serum at 20 % (v/v) concentration for 10 minutes at room temperature.
4. This was followed by incubation at room temperature with one of the 4 primary antibodies used in this study and left overnight.
5. All slides incubated with primary antibodies were then washed with TBS and incubated with biotinylated rabbit anti-mouse immumoglobulins at 1:200 dilution for 30 minutes at room temperature.

6. After washing all the slides in TBS, incubation of all slides in streptavidin-peroxidase at 1:400 dilution for 30 minutes at room temperature.

7. The slides were washed again by TBS and the color development was done using 3', 3' - diaminobenzidine tetrahydrochloride (DAB) or 3 - amino -ethylcarbazole (AEC)

8. All slides were then rinsed gently with distilled water, counterstained with Mayer's hematoxylin for 15 minutes, washed in running water, dehydrated, cleared and mount.

9. The number of positive cells were obtained by counting in each specimen. Damaged cells or cells without nuclei were not counted.

Controls:

Positive controls: optic nerve was used as a control for neuron retinal cells in subretinal fluids, while liver section was as a control for macrophages. Lymphaden sections were used as a controls for T and B cells. Positive controls were run simultaneously during each immunohistochemical staining.

Negative controls: negative controls were run according to the steps described in the immunological procedures except that TBS buffer was substituted for the primary antibody.

3.4.4 Examination of autofluorescence

Autofluorescent examination of retinal pigment epithelium in subretinal fluid was carried out using a Zeiss Microscope Photometer System (Fig 3.2). The system consisted of a Zeiss universal microscope with fast scanning stage, neofluar objectives, epi-fluorescence Condenser III RS and phase-contrast illumination.

To assess the nature of pigment granules in iris and ciliary and retinal pigment epithelial cells, 5 um thick whole-eye frozen sections were prepared from five eyes obtained by autopsy in five subjects without any known ocular disease. Autofluorescent analyses were done on these sections.

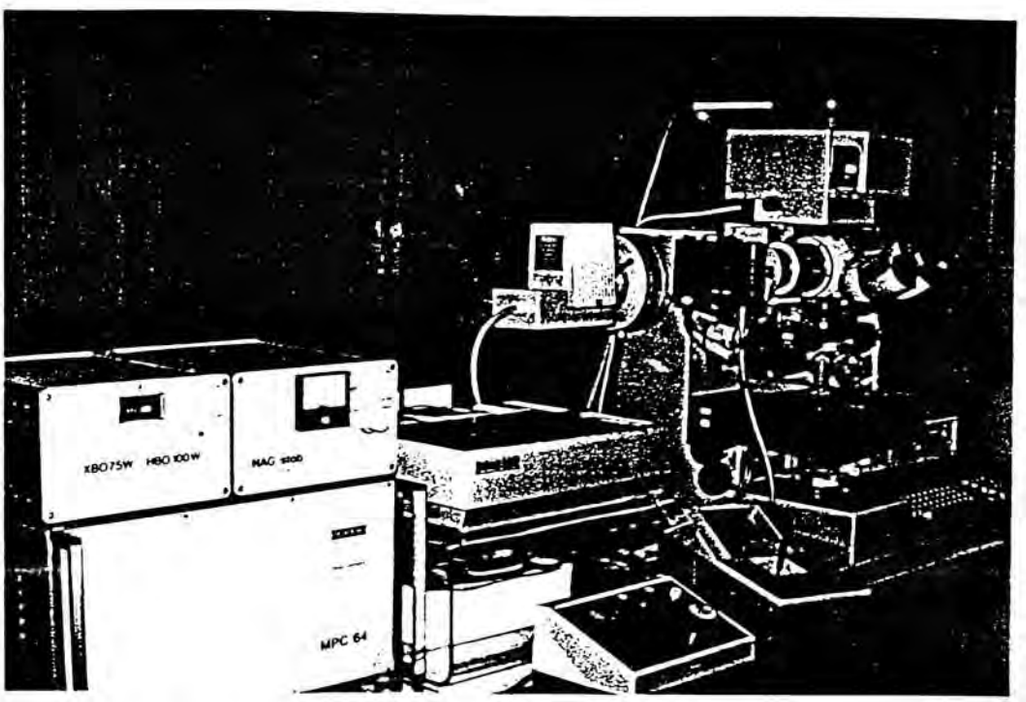

Fig 3.2 This consists of photograph showed Zeiss microscope photometer system: universal microscope with the fast scanning stage, neofluat objectives, epi-fluorescence condenser 3 RS, phase-contrast illumination, light source (HBO 100w/2) and the microscope photometer control MPC- 64 unit.

3.4.5. Hematoxylin and eosin staining

Cytologic examination of subretinal fluids was prepared according to the following steps:

1. Sections were fixed in 90% ethanol for 30 min,
2. Section were transferred to 70% and 50% ethanol respectively, each for 10 minutes.
3. Tap water was used to rinse until water run off evenly,
4. Stain in hematoxylin (Mayer's) for 15 minutes,
5. Rinsed in tap water twice 10 dips each,
6. Dipped in 0.25% ammonia water, until blue color appeared.
7. Rinsed in tap water twice10 dips each.
8. Stained in 1% Eosin for 10-20 minutes dips.
9. 95% ethanol for 10 minutes, twice,
10. Transferred to absolute ethanol, with three changes and 10 minutes each, charge,
11. Three changes in xylene 10 minutes for each, charge,
12. The slides were mounted for observation.

CHAPTER 4

RESULTS

4.1 Total protein

4.1.1 Standard curve of protein determination

Using crystalline bovine serum albumin with Lowry's method, a standard curve of protein determination was obtained as below (Fig.4.1).

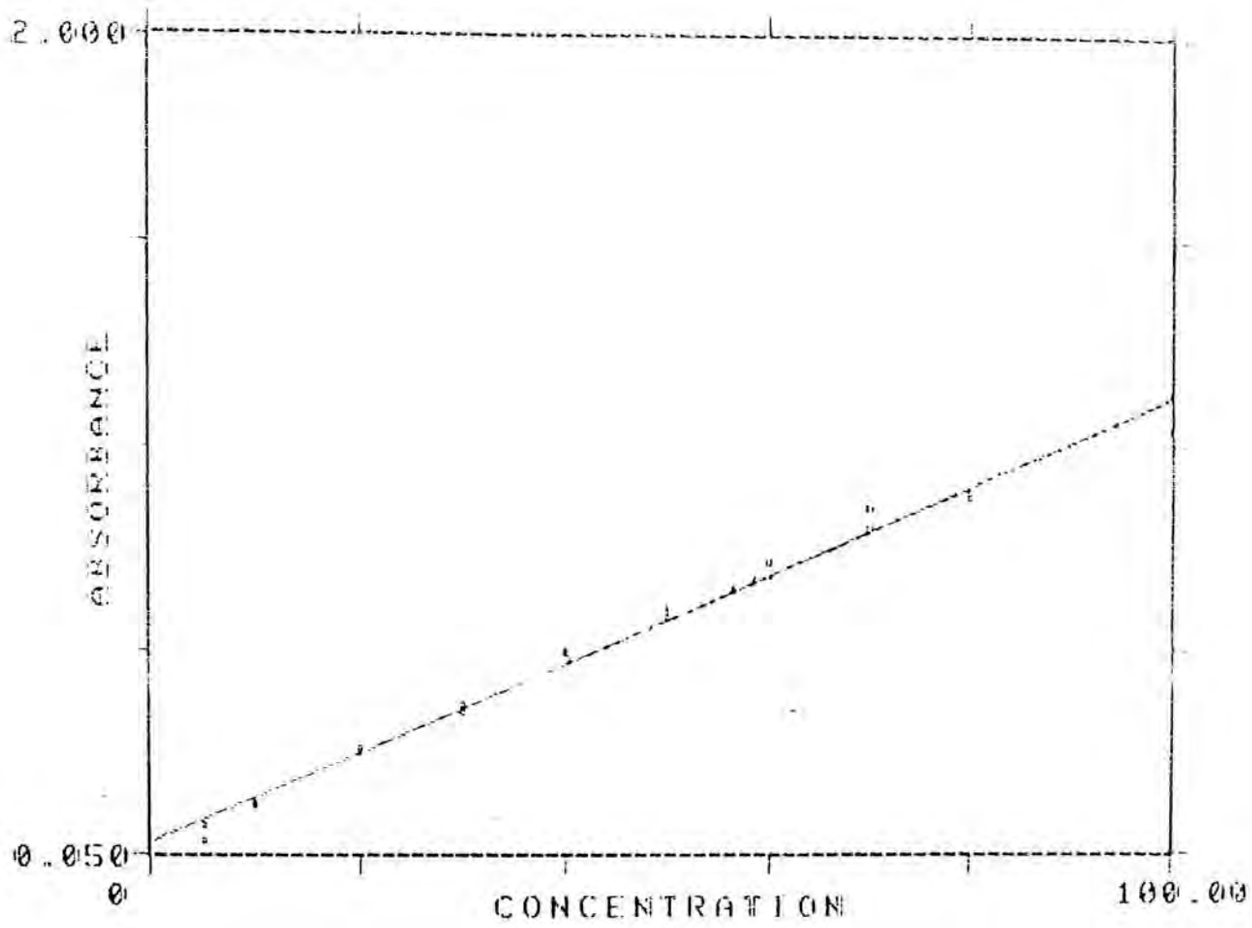

Fig. 4.1 Standard curve of protein determination

4.1.2 Total protein in subretinal fluids

46 specimens of subretinal fluid were collected. Information about each case is given in Table 4.1.

Table 4.1 Date of cases from 46 specimens of subretinal fluid were obtained for analysis

Case No.	Age/ Sex	Lens removed	PVR	Hole number	Hole size (DA*)	Duration (mons)	Protein (mg/ml)
1	35/M	-	D3	0	_	12	**71.4**
2	14/M	-	<C1	1	1/4	9.5	**71.0**
3	12/M	-	<C1	1	1/3	2.5	**67.88**
4	76/F	-	D2	1	1/4	4	**62.44**
5	65/F	-	D1	1	1/4	6	**60.3**
6	43/M	-	D3	1	1/3	12	**56.03**
7	40/M	-	D2	1	2/3	4	**54**
8	43/M	-	D3	2	_	?	**52.5**
9	72/M	+	D3	1	1	9.5	**50.6**
10	51/M	-	C1	1	1	4	**49.9**
11	42/M	+	C3	1	1/5	5	**48.23**
12	39/F	-	D3	1	2/3	8	**48.1**
13	70/M	-	C3	1	1/4	5	**47.6**
14	60/M	-	D3	1	1/3	2	**45.9**
15	74/F	-	D1	1	1/4	3.5	**41.57**
16	32/M	+	D3	1	1/4	2	**39.5**
17	65/F	-	D1	1	1/4	3	**39.28**

continue...

18	33/F	-	C3	1	3/4	5	**36.37**
19	44/F	-	D2	1	1/4	2	**35.95**
20	78/M	+	D3	1	1	3	**34.4**
21	75/M	+	D2	1	1/3	2	**34.08**
22	35/M	-	C3	0	_	6	**32**
23	37/M	-	D3	1	1/4	6	**29.59**
24	27/M	+	D3	2	_	?	**29**
25	27/F	-	<C1	1	1/3	2	**26.48**
26	11/M	-	D3	2	_	6	**26.155**
27	71/M	-	D2	2	_	2	**25.7**
28	54/F	-	D3	1	1	?	**25.2**
29	82/M	-	C3	1	1/4	4	**24.86**
30	29/M	-	D1	1	1/4	3	**23.3**
31	46/F	+	D2	1	1/2	2	**22.35**
32	62/M	-	D2	1	1/4	2	**21.9**
33	50/M	-	D3	1	1	3	**20.6**
34	31/M	+	D1	1	2	1	**20.1**
35	45/M	+	D2	1	1/3	3	**19.7**
36	27/M	+	D3	1	3	4	**16.94**
37	46/M	-	D1	1	2	1.5	**15.9**
38	59/M	-	<C1	1	1/3	2	**14.7**
39	65/F	-	<C1	1	1/2	1.5	**10.14**
40	50/M	-	<C1	1	giant	0.5	**4.5**
41	60/M	+	<C1	4	_	?	**3.2**
42	68/F	-	D1	1	1 1/4	1	**2.6**
43	32/M	-	C3	1	2	2	**0.657**
44	50/M	-	D2	1	1/3	1	**0.375**
45	29/F	+	D1	3	_	?	**0.33**
46	27/F	-	C3	1	giant	?	**0.23**

* DA = disc area

4.1.3 Total protein in normal vitreous of autopsy and sera of patients

The levels of normal vitreous obtained from 11 autopsy were shown to be ranged from 0.23-0.35 mg/ml. The protein contents of sera from 12 patients with retinal detachment ranged from 61.2-72.7 mg/ml.

4.1.4 Relationship of protein level and size of retinal break

The total protein content of subretinal fluid in 46 cases were presented in Table 4.1. Of the 46 cases, 38 have one retinal break, 2 patients with no break. The remaining 6 cases were found to have more than one break and were not included. All the 40 cases with one break or no break were divided into 3 groups. The size of retinal break was grouped as below: (1) more than 1 disc area (DA); (2) between 1/3 - 1 DA; (3) less than 1/3 DA (including the cases of no break). Figure 4.1 showed a graph of mean total protein content plotted against the size of retinal break. A P value less than 0.0001 was obtained by F test, i.e. significant. It seems that the larger the size of retinal break, the lower the total protein level (Fig.4.1).

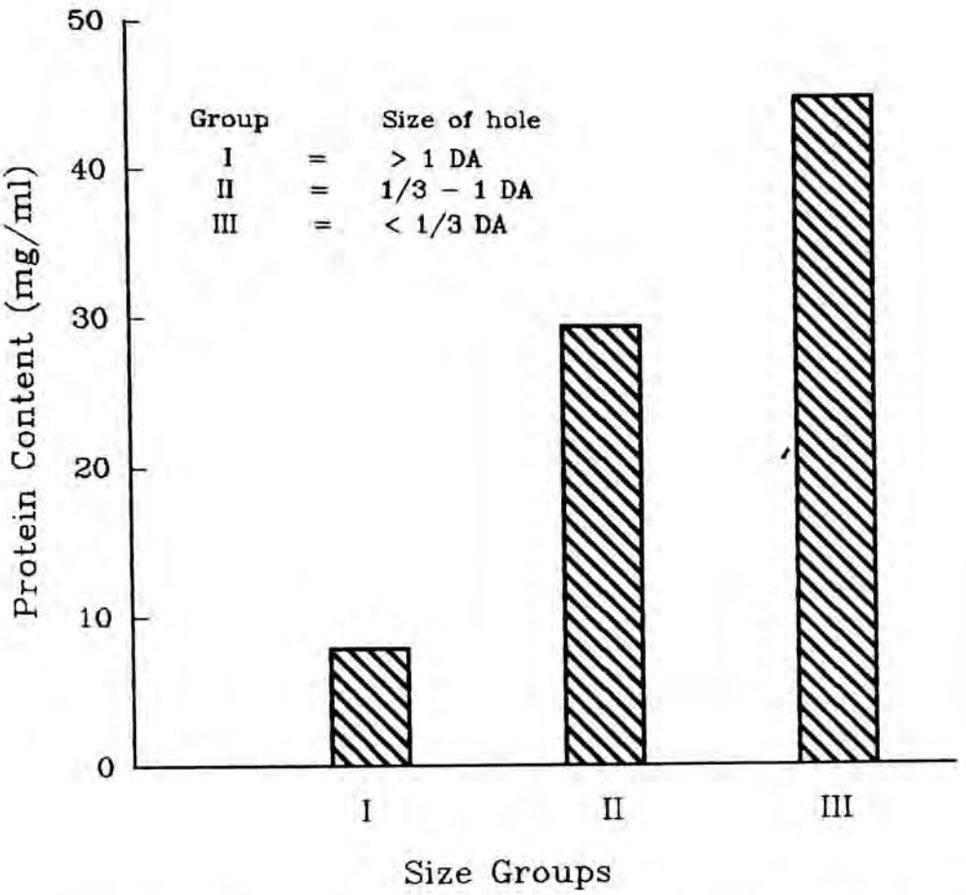

Fig 4.1 Average measurements of protein content in subretinal fluid among different size groups of retinal hole ($P < 0.0001$).

STATISTICS

The data was analyzed by SPSS/PC + program:

Value label	Mean	Std Dev	Sum of sq	Cases
Group 1	7.9071	8.3290	485.6060	7
Group 2	29.4938	16.9367	4015.9406	18
Group 3	45.1889	16.1828	4452.0322	15
Total	32.1723	15.3499	8953.5788	40

Analysis of Variance

Source	Sum of squares	D.F.	Mean square	F	Sig
Between groups	7867.7737	2	3933.8869	16.6959	**0.0000**
Within groups	8953.5789	38	235.6205		

eta = .6839 eta squared = .4677

4.1.5 Relationship of protein level and duration of retinal detachment

As seen from Table 4.1 and Fig. 4.2, the protein contents of subretinal fluids in 40 cases increased with the duration of retinal detachment.

Figure 4.2 showed the graph of total protein contents plotted against duration of retinal detachment. By means of linear regression analysis and F-test regression equation, P value of less than 0.0001 was calculated.

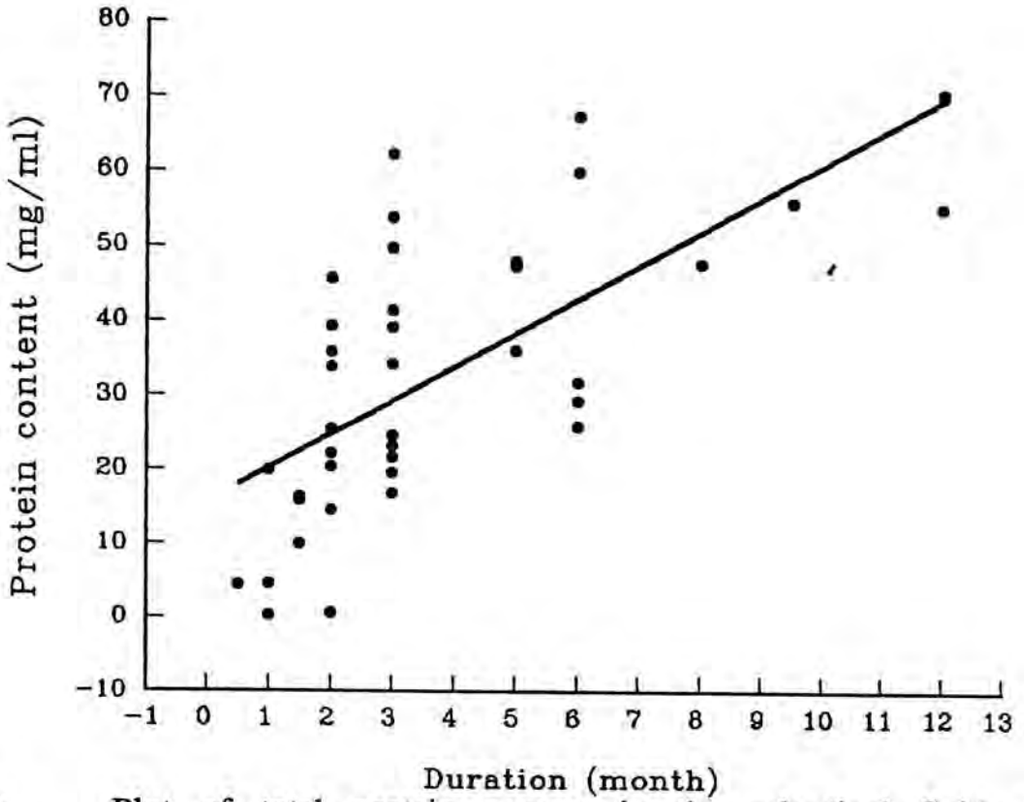

Fig 4.2 Plot of total protein content in the subretinal fluid against duration of retinal detachment ($p<0.0001$)

STATISTICS

The data was analyzed by SPSS/PC+ program

Multiple regression

Multiple R	.72943
R square	.53207
Adjusted R square	.51976
Standard Error	13. 49433

Analysis of variance (F test of regression equation)

	DF	Sum of squares	Mean square
Regression	1	7868.18615	7868.18615
Residual	38	6919.68182	182.09689
F=43.20879	signif F=0.000		

Variable in the equation

Variable	B	SEB	Beta	T	Sig T
Duration(m)	4.69728	.71460	.72943	6.573	**.0000**
(constant)	14.39119	3.62442		3.971	.0003

4.1.6 The relationship of protein level and degree of PVR

46 cases were divided into three groups according to the severity of PVR: less than grade C1; grades C1-C3; and grades D1-D3.

Figure 4.3 depicted total protein contents of subretinal fluids plotted against the various degree of PVR. Using F test, a P value of more than 0.5 was calculated.

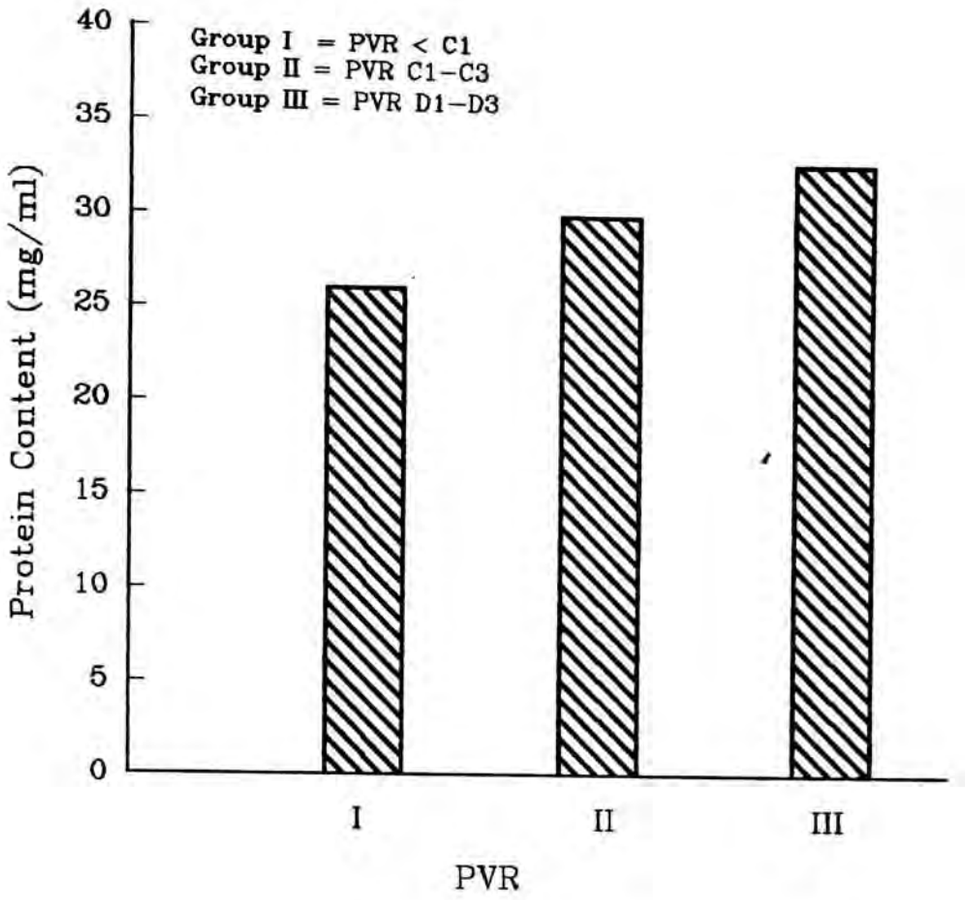

Fig. 4.3 Average measurements of protein content in subretinal fluids among various grades of PVR (P=0.6971).

STATISTICS

The data was analyzed by SPSS/PC+ program:

Value label	Mean	Std Dev	Sum of Sq	Cases
group 1	26.0000	27.4270	5265.6944	8
group 2	29.9809	20.2003	2856.3610	8
group 3	32.9127	19.0847	10562.5609	30
total	31.2006	20.8453	18684.5609	46

Analysis of Variance

Source	Sum of squares	D.F.	Mean Square	F	Sig
Between groups	316.2069	2	158.1036	.3639	**.6971**
With groups	18684.6164	43	434.5260		

eta=.1290 eta squared=.0166

4.2 Basic FGF in subretinal fluids

4.2.1 Standard curve for determination of bFGF in subretinal fluid.

Using purified recombinant human bFGF, a standard curve with bFGF ranging from 10 pg/mi to 640 pg/ml was prepared (Fig 4.4).

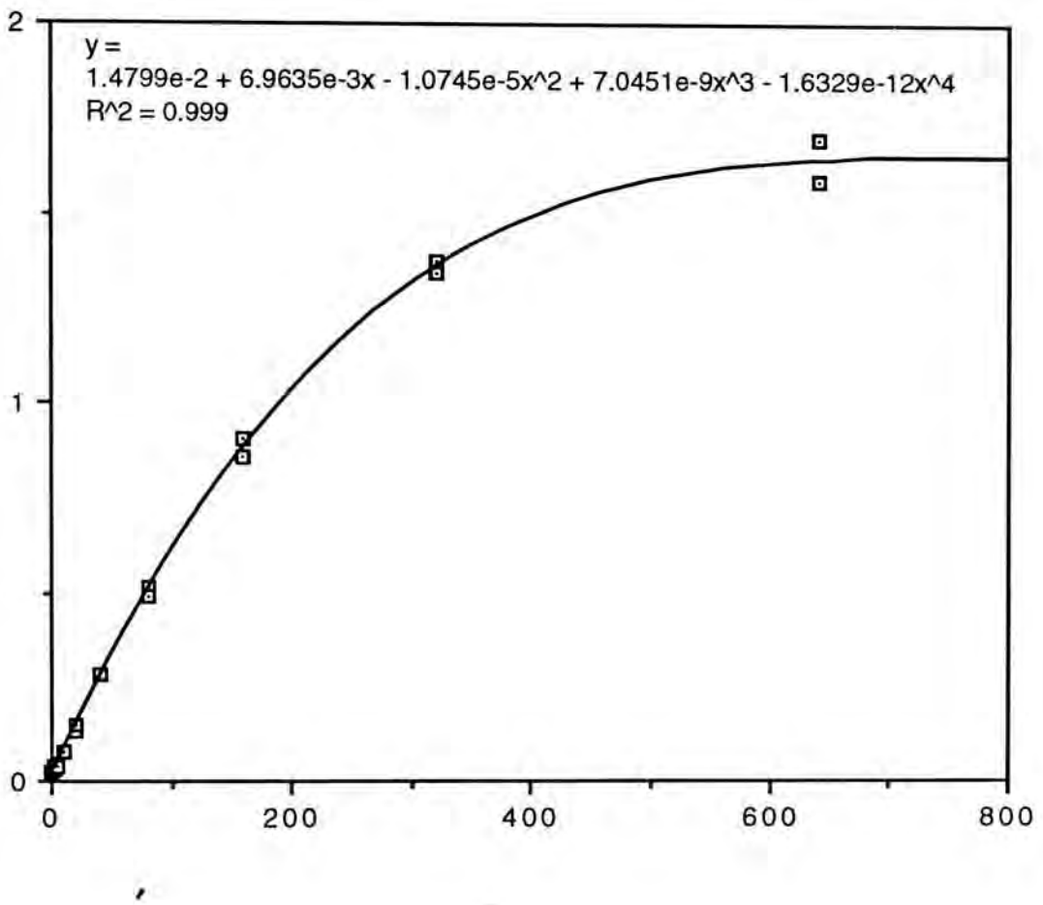

Fig 4.4 Concentration of bFGF (pg/ml)
(basic FGF standard curve)

4.2.2 The levels of bFGF in both subretinal fluids and controls

The concentration of bFGF in subretinal fluids was calculated by comparing the optical density of aspirates with that of a standard curve (Table 4.5).

Table 4.5 Patient data:

Patient no.	Age/Sex	Pre-cryo	Grade of PVR	bFGF (pg/ml)	Protein* (mg/ml)
1	70/F	+	D3	**260**	
2	74/F	-	D1	**256**	41.57
3	39/F	+	D3	**230**	48.1
4	60/M	+	D3	**201**	45.9
5	11/M	+	D2	**197**	26.2
6	71/M	+	D2	**192**	25.7
7	72/M	-	D3	**182**	50.6
8	31/M	-	D1	**172**	20.1
9	27/M	-	D3	**167**	29.0
10	46/F	-	D2	**161**	22.35
11	29/F	-	D1	**157**	0.33
12	50/M	-	D2	**157**	0.375
13	22/M	+	D2	**156**	
14	32/F	-	D3	**155**	39.8
15	46/M	-	D1	**152**	15.9
16	27/M	-	D3	**150**	16.94
17	78/M	-	D3	**150**	34.4
18	68/F	+	D1	**148**	4.6
19	35/M	+	D3	**144**	71.4
20	40/M	+	D2	**144**	54

continue ...

21	37?M	-	D2	**144**	29.59
22	44/F	-	D2	**142**	35.95
23	30/F	+	D3	**142**	
24	39/M	-	D2	**141**	
25	23/M	+	C3	**140**	
26	76/F	+	D2	**140**	62.44
27	75/M	-	D2	**128**	34.8
28	65/F	-	D1	**119**	60.3
29	32/M	+	C3	**118**	0.657
30	35/M	+	C3	**79**	32
31	70/M	-	D3	**75**	47.6
32	61/M	-	D3	**64**	
33	27/F	-	C3	**64**	0.23
34	46/M	+	D3	**60**	
35	12/M	-	<C1	**50**	67.88
36	65/F	-	<C1	**47**	10.14
37	33/F	-	C2	**44**	36.37
38	14/F	-	<C1	**39**	7.1
39	50/M	-	<C1	**36**	4.5
40	59/M	+	<C1	**30**	14.7
41	82/M	-	C3	**24**	24.86
42	27/F	-	<C1	**23**	16.48
43	60/M	-	<C1	**0**	3.2

* Protein contents: determined by the Lowry's method.

Basic FGF was undetectable in all serum samples and in 2 of the 10 normal autopsy vitreous. Low levels of bFGF ware detected in the remaining 8 autopsy vitreous (Table 4.6).

Table 4.6 Basic FGF in autopsy vitreous:

No. of autopsy	bFGF (pg/ml)
1	27
2	16
3	13
4	11.5
5	11
6	10
7	8
8	5
9	0
10	0

4.2.3 Levels of bFGF in different degrees of PVR

Table 4.7 summaries the levels of bFGF in subretinal fluids obtained from patients in the present study and the bFGF levels are grouped according to the severity of PVR.

Table 4.7 Basic FGF levels in different diagnosis groups

Group	Number of Samples	Mean bFGF
PVR<C	7	32.2 ± 16.9
PVR C*	9	70.3 ± 26.6
PCR D**	27	160.1 ± 35.1

*PVR C group is significantly different from the PVR<C group, P<0.01(student t-test)
**PVR D group is significantly different from the PVR C group, P<0.001(student t-test)

Subretinal fluids obtained from eyes with PVR ≥ C contain more than 4 times the amount of bFGF (141.0 ± 35.7 pg/ml) as that found in eyes of the group with PVR<C1 (32.2 ± 16.9 pg/ml) (see Fig 4.5).

Detailed analysis of bFGF concentrations among patients with PVR<C1, PVR C, PVR D was depicted in Figure 4.6.

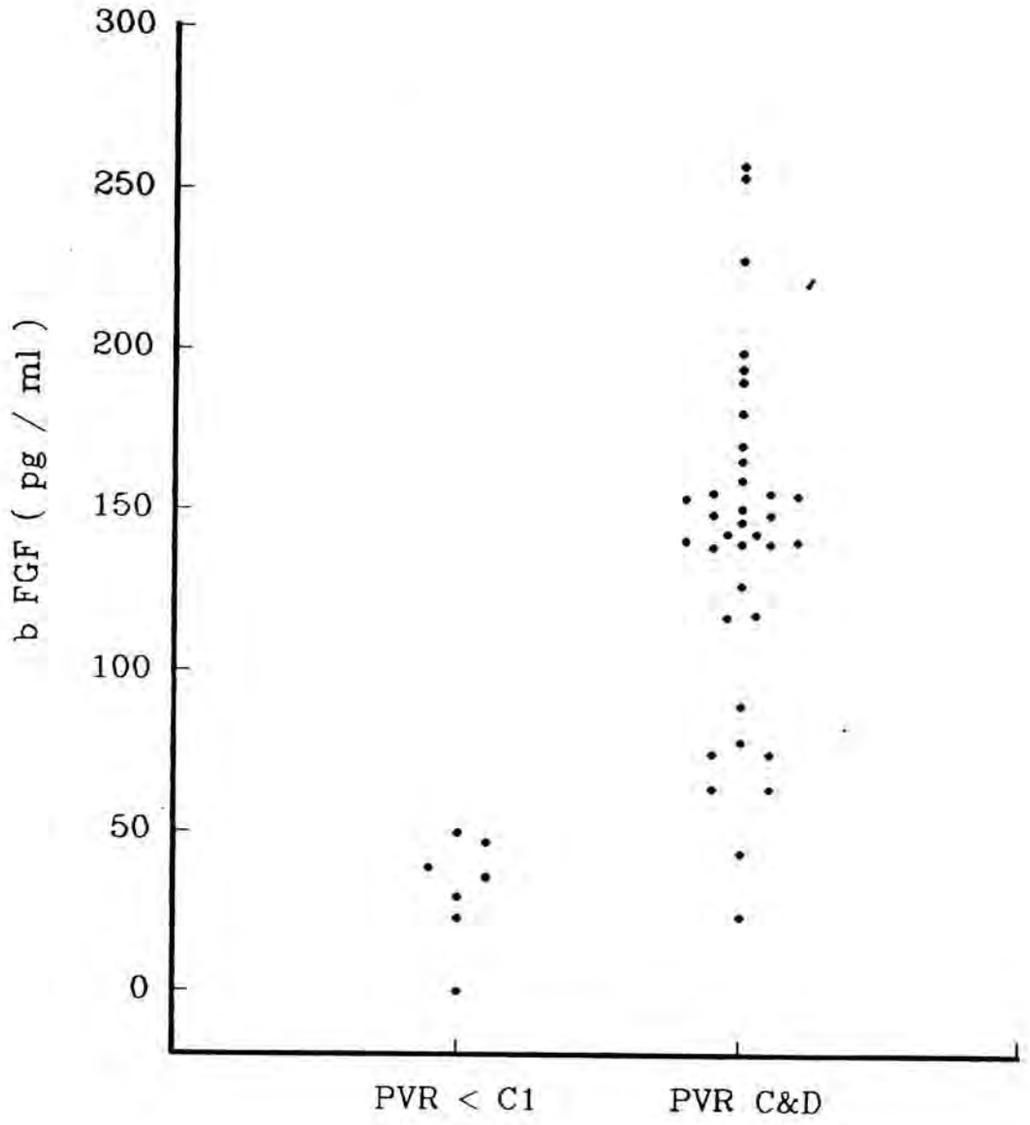

Figure. 4.5 Quantitation of the bFGF levels of subretinal samples. Subretinal samples were grouped according to the PVR < C1 or PVR ≥ C1 ($P<0.001$, *t*-test)

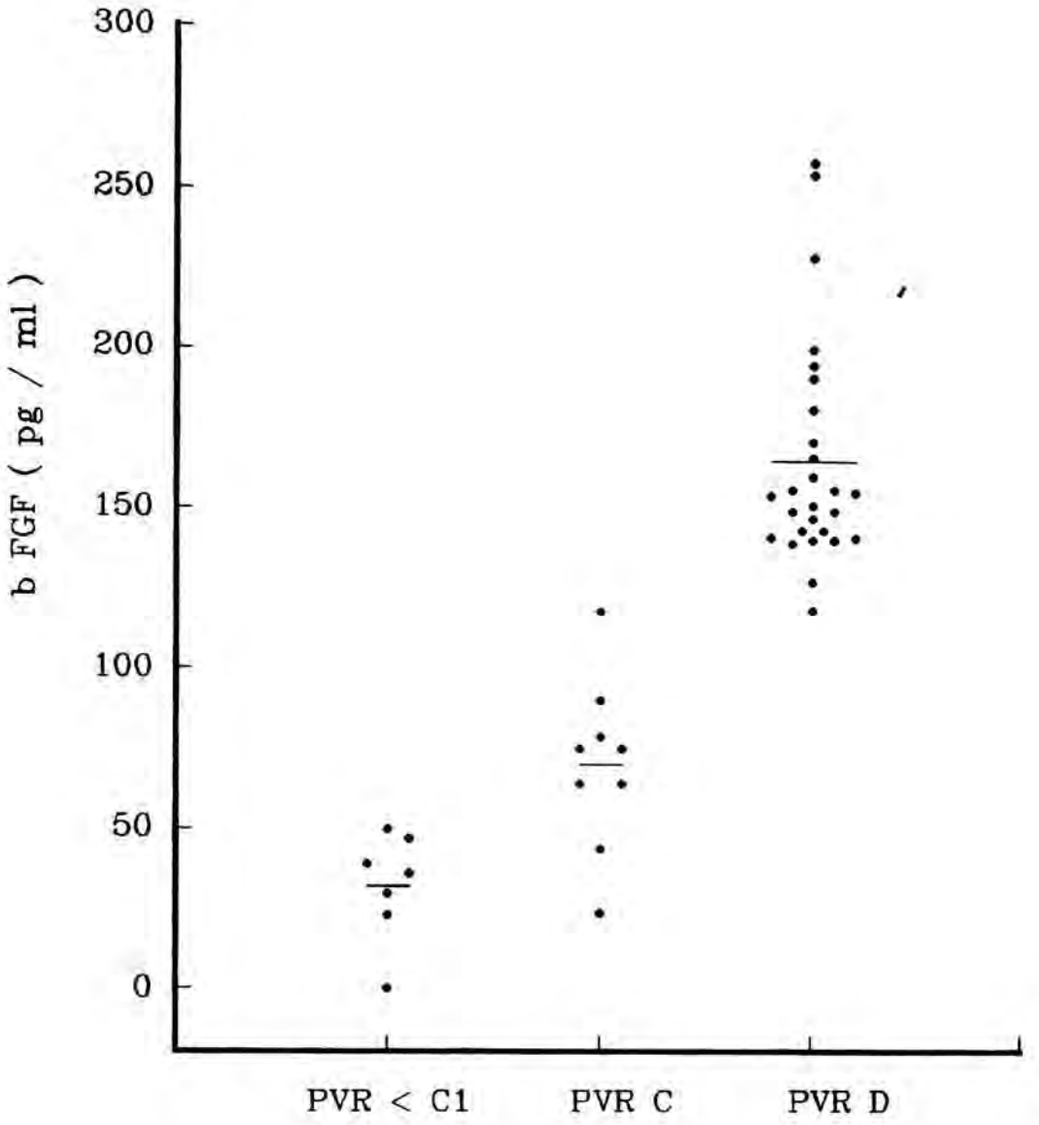

Figure 4.6 Quantitation of the bFGF levels of subretinal fluids. Subretinal fluids were grouped according to severity of PVR. Horizontal bars denote median values.
PVR C group different from the PVR<C group, $P<0.01$ (student *t*-test); PVR D group different from the PVR C group, $P<0.001$ (student *t*-test)

4.2.4 Levels of bFGF in subretinal fluids of retinal detachment with and without previously cryotherapy

Subretinal fluid samples collected from patients with PVR C and D were grouped into two groups. The first group consisted of 16 patients who had been treated previously by the method of cryotherapy, but the second group consisted of 11 patients who received no previously cryotherapy. Nine patients who had received other eye operations previously, such as cataract surgery, vitrectomy, etc. were not included for analysis.

The levels of bFGF in the group of sixteen patients with previous cryotherapy were found to be higher, i.e. mean bFGF = 90 ± 19.3 pg/ml; n = 4 (PVR C) and mean of bFGF = 184.0 ± 45.2 pg/ml; n = 12 (PVR D) than the group of 11 patients without previous cryotherapy i.e. mean bFGF = 54.2 ± 20.55 pg/ml, n = 5 (PVR C) and mean bFGF = 138.0 ± 12.8 pg/ml, n = 6 (PVR D) respectively, $P < 0.05$ (*t* test) (see Table 4.8).

Table 4.8 Basic FGF levels by therapeutic group

Diagnosis	Pre-cryo-therapy	Number of samples	Mean bFGF	Student *t*-test
PVR C	yes	4	90.5 ± 19.3	
				P<0.05
PVR C	no	5	54.2 ± 20.55	
PVR D	yes	12	184.0 ± 45.2	
				P<0.05
PVR D	no	6	138 ± 12.8	

4.2.5 The relationship of levels of protein and bFGF

In 36 cases both total protein and bFGF were measured. The relationship of levels of protein and bFGF was analysed in Table 4.5.

Figure 4.7 showed the plot of bFGF concentration of each specimens against total protein level. By means of linear regression equation, a P value of more than 0.3 was calculated.

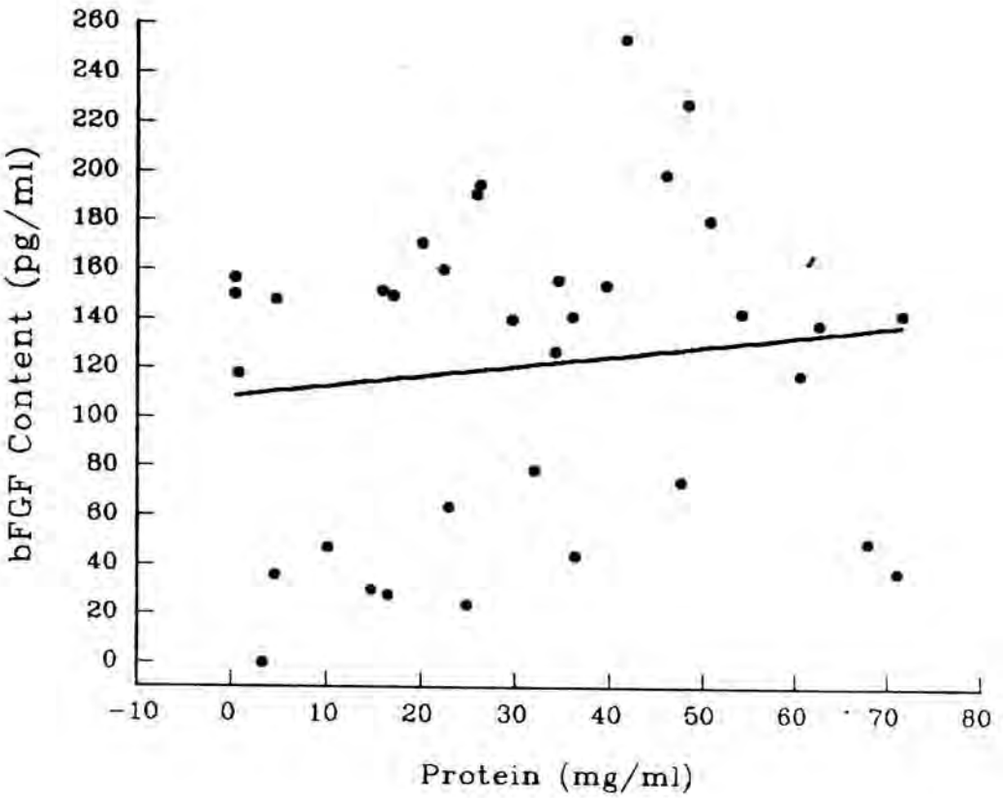

Fig 4.7 Basic FGF contents plotted as protein levels of subretinal fluids. Scatter diagram and regression line ($P > 0.3$)

STATISTICS

The data was analyzed by SPSS/PC+ program

Multiple regression

Multiple R	.16317		
R square	.02662		
Adjusted R square	-.00200		
Standard Error	64.205		
Analysis of variance	DF	Sum of squares	Mean square
Regression	1	3833.65723	3833.65723
Residual	34	140158.56499	4122.31074

F=.92998 signif F=.3417

Variables in the equation

Variable	B	SEB	Beta	T	Sig T
Protein	.48722	.50523	.16317	.964	.3417
(Constant)	107.52044	18.62593		5.773	.0000

4.3 Results of cytological examination

4.3.1 Pigment examination by autofluorescence

Pigment examination on normal eye sections exhibited autofluorescence, i.e. a sharp delimitation was seen between retinal pigment epithelium (continuous and strong autofluorescence, Fig 4.8a) and ciliary or iris pigment epithelial cells were totally devoid of lipofuscin. The latter was therefore not autofluorescence (Fig. 4.8 b). Only a few granules of lipofuscin were seen in pigment epithelial cells of pars plana adjacent to the peripheral retina. On the basis of this feature, retinal pigment epithelium from other pigment epithelial cells in subretinal fluid samples can be distinguished.

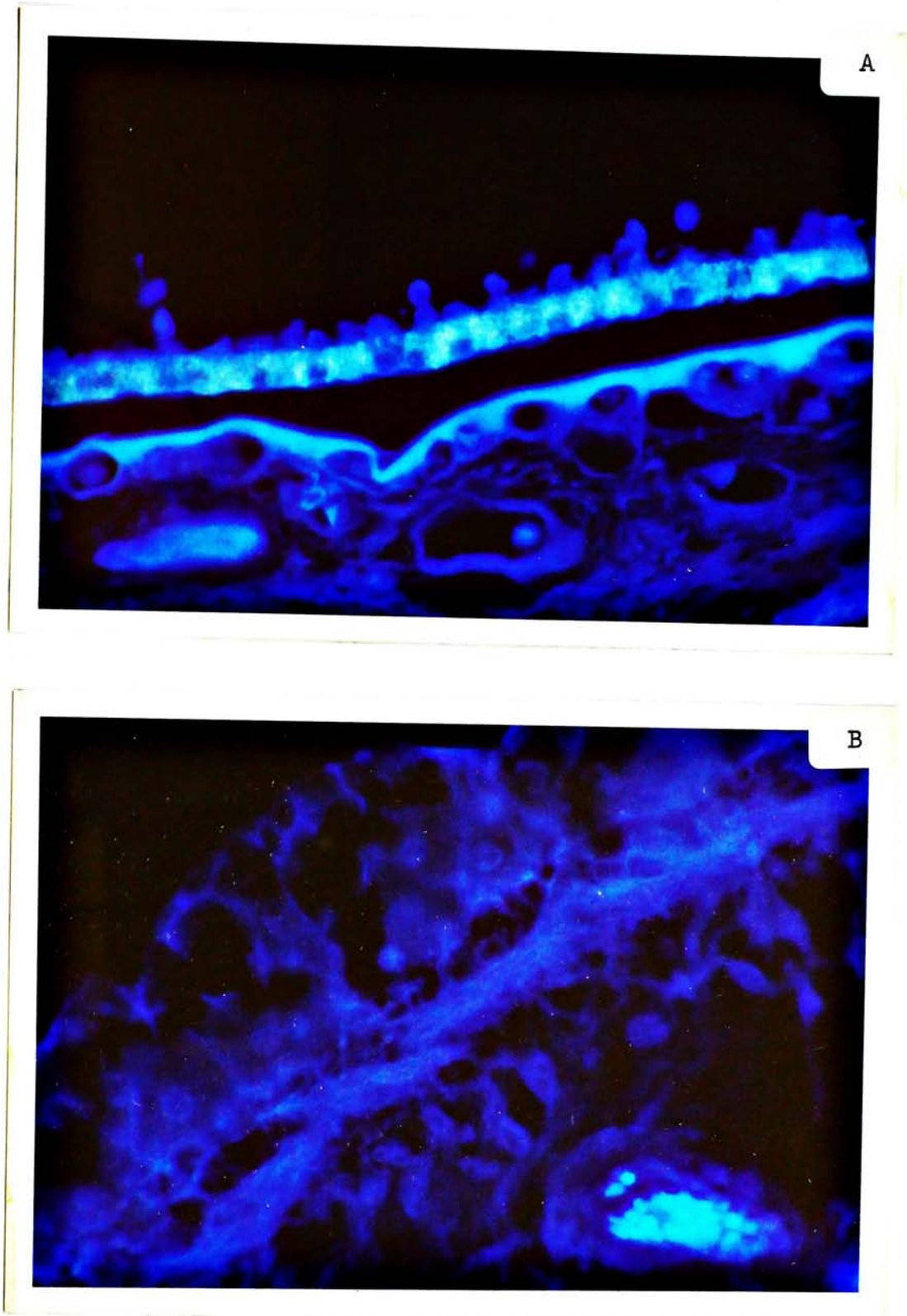

Fig. 4.8 Normal eye sctions showing the bright continuous autofluorescence of retinal pigment epithelium (A) and the iris pigment epithelial cells were devoid of lipofuscin (non-autofluorescence (B) (original magnification x 400).

4.3.2 Cellular study of subretinal fluids

Cytological examination of subretinal fluids specimens showed four major cellular patterns.

1) Neuron retinal cells. These cells were observed in 23 of 35 specimens. Their shapes varied according to the origin of the cell and the duration of retinal detachment. Some cells appeared oval in shape with the eccentric nuclei on one side, while on the other side, the cytoplasm showed some shrinkage. The morphology of these cells suggests that they are neuroepithelial cells. The origins of these cells ware confirmed by the immunoperoxidase staining method (Fig.4.9)

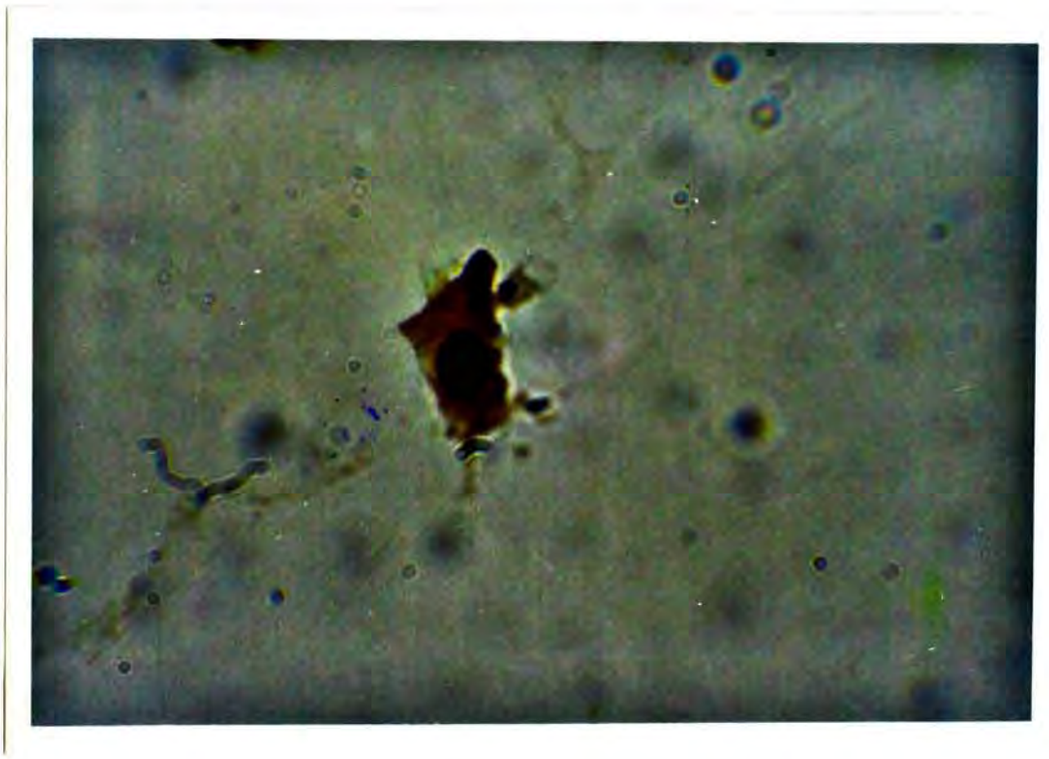

Fig. 4.9 Immunoperoxidase reactivity of Anti-Human Neuron-Specific enolase antibodies in a neuron retinal cell (original magnification x 1000)

2) Heavily pigmented cells. This cell type appeared in all 35 cases. The cytoplasm was filled with pigment granules and were totally devoid of lipofuscin and contained only nonautofluorescent pigment granules. The nucleus was covered by the numerous pigment granules (Fig. 4.10).

Fig 4.10 Heavily pigmented cells containing only melanin which masks the nucleas (x 400)

3) Retinal pigment epithelium. These cells appeared either isolated or in groups and were found in all 35 specimens. They were rich in lipofuscin granules which were strongly autofluorescence (Fig. 4.11). In long-standing detachment cases, however, melanin containing RPE cells were hardly seen.

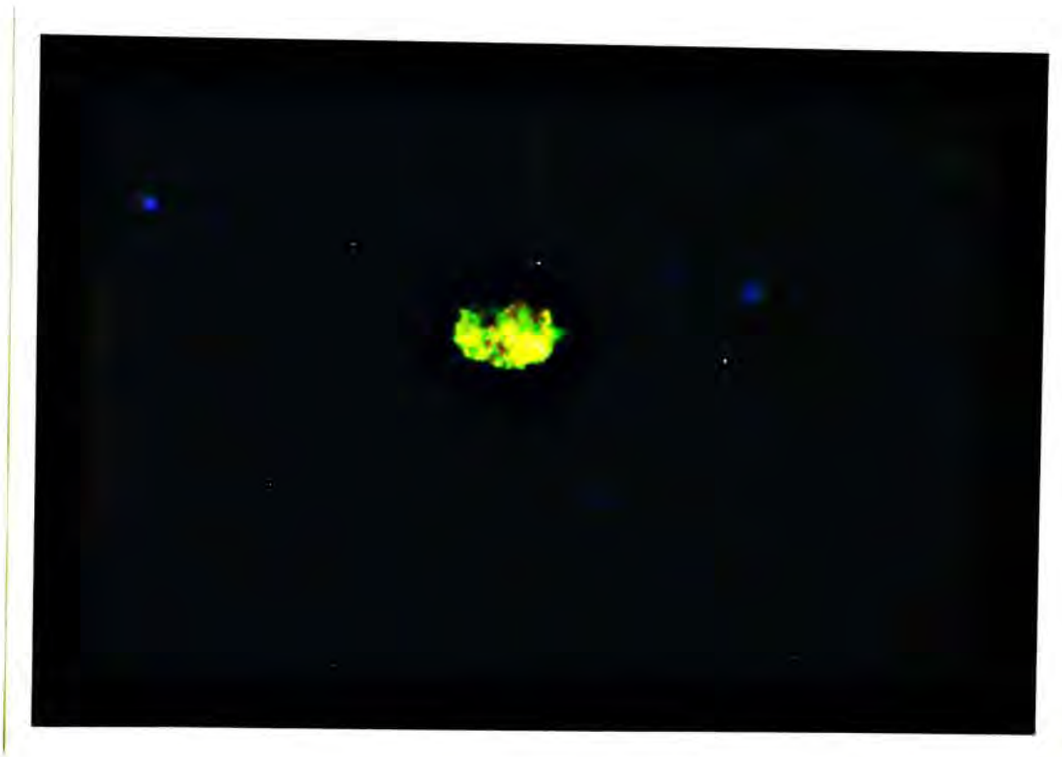

Fig. 4.11 Analysis of pigment by epi-illumination showed this with lipofuscin, supposed to originate from the retinal pigment epithelium (original magnification x 400)

4) Macrophage were seen in 16 of 35 subretinal fluid specimens. The cells showed either a common reniform indented nucleus with a homogeneous, sharply marked cytoplasm or a vacuolated cytoplasm with pigmented granules and an indistinct cell boundary. The latter morphology could be linked to a regressive change caused by a long period of bathing in the subretinal fluid. These cells were found to be positive by immunostaining using antibodies, specific to human myeloid /histiocyte antigen (Fig 4.11).

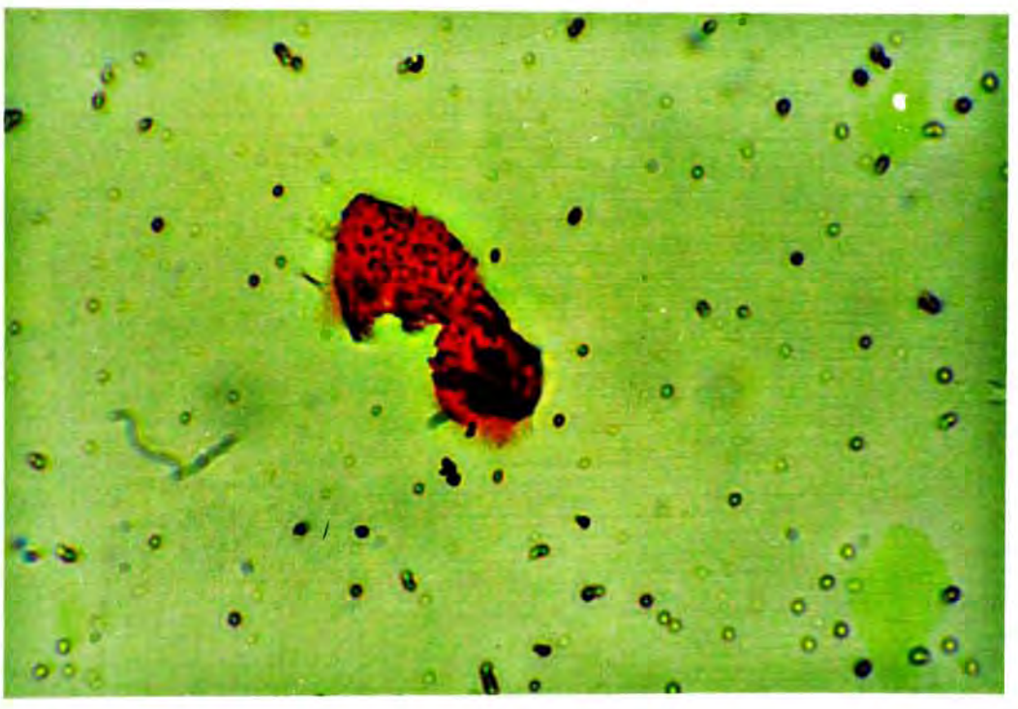

Fig. 4.12 Macrophage cell. Only a few pigment granules were seen within the cytoplasm, Using immunocytological procedure, this kinds of cells were positive for macrophage markers. (magcificantion x 1,000).

5)Lymphocyte was not seen in our subretinal fluid specimens. Two anti-lymphocyte markers, anti-human T cells and anti-human B cells, were found to be negative.

4.3.3 Cellular study of subretinal fluid in eyes with prior cryotherapy

Immunocytology, autofluorescent analysis and H&E staining were carried out in 20 specimens (PVR C and D) (Table 4.9, 4.10). In addition, bFGF levels of these specimens were also determined. The results of cytologic examination of SRF showed no difference between cell types seen from patients who previously underwent cryotherapy and those without previous cryotherapy. However, marked difference was observed in these two groups of patients in terms of the cell numbers and the presence of the severely damaged cells.

Table 4.9 Cellular study of subretinal fluid in eyes with prior cryotherapy

No. of patient	Grade of PVR	RPE cells	Neuro-retinal cells	Dispersive lipofuscin
1	C	+	++	+
2	C	+++	+++	++'
3	D	++	++	++
4	D	++	+	++
5	D	+	+++	++++
6	D	+++	+++	+++
7	D	++	++++	+++
8	D	+++	++++	++++
9	D	++	++++	+++
10	D	++	++++	+++

*One plus denotes less than 50 cells per slide. Two pluses denote 50 to 100 cells per slide. Three pluses denote greater than 100 cells per slide. Four pluses denote large sheets of cells detected on one slide. The number of dispersive lipofuscin were only estimated from one plus to four pluses.

Table 4.10 Cellular study of subretinal fluid in eyes without cryotherapy

No. of patient	grade of PVR	RPE cells	Neuro-retinal cells	dispersive lipofuscin
11	C	+	-	-
12	C	+	-	-
13	C	+	-	+
14	C	+	+	-
15	D	+	+++	+++
16	D	+	+	+
17	D	+	-	+
18	D	+	+	+
19	D	+	++	+
20	D	++	+	+

*One plus denotes less than 50 cells per slide. Two pluses denote 50 to 100 cells per slide. Three pluses denote greater than 100 cells per slide. Four pluses denote large sheets of cells detected on one slide. The number of dispersive lipofuscin were only estimated from one plus to four pluses.

Large amounts of neuron retinal cells and fragments removable from the subretinal space by aspiration were detected in 9 specimens (Fig.4.13). Of the 9 cases studied, 8 of 9 were found in specimens of patients with previous cryotherapy. Limited and isolated neuroretinal cells were found in 7 specimens. No neuroretinal cell was detectable in other 4 specimens obtained from patients without previous cryotherapy.

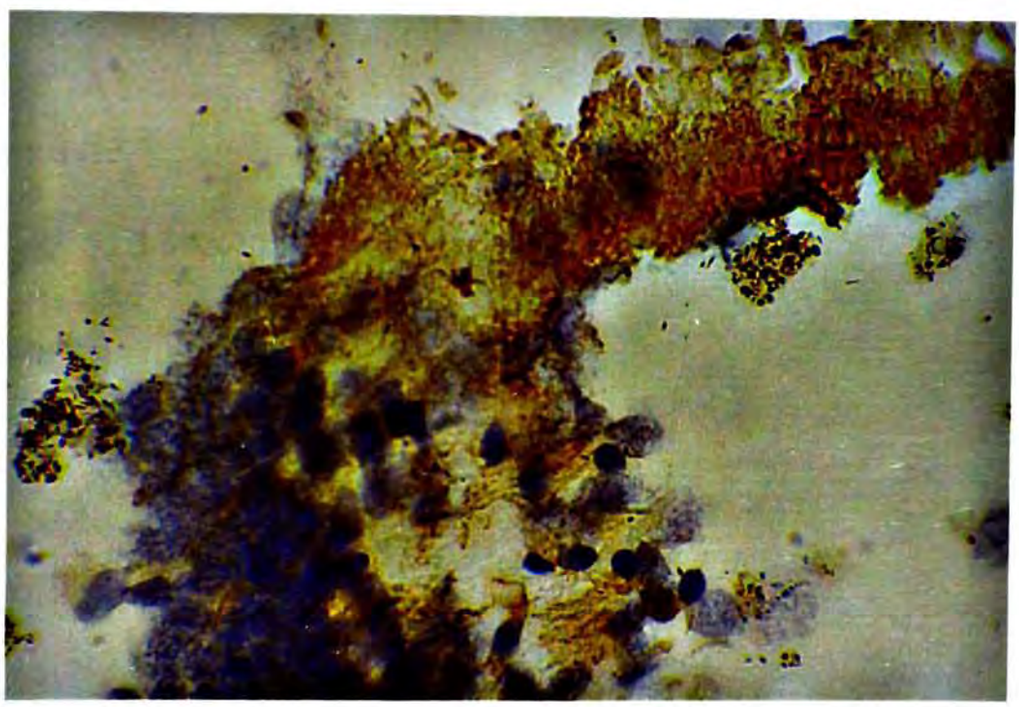

Fig. 4.13 Specimen from a patient suffering from retinal detachment with previous cryotherapy. Immunoperoxidase reactivity of a large sheet of neuroretinal cells with NSE antibodies. Nucleus is counterstained in blue by haematoxylon (x 400).

Intact RPE cells were shown in all specimens by H&E staining and autofluorescent analysis. An increase in number of RPE cells was found in most specimens of patients with previous cryotherapy (Fig 4.14).

The presence of a large number of dispersive lipofuscin suggested that many RPE cells were disintegrated (Fig. 4.15). This phenomenon occurred in most of specimens obtained from patients with previous cryotherapy. In specimens obtained from patients without previous cryotherapy, dispersive lipofuscins were found to be scarce or negative.

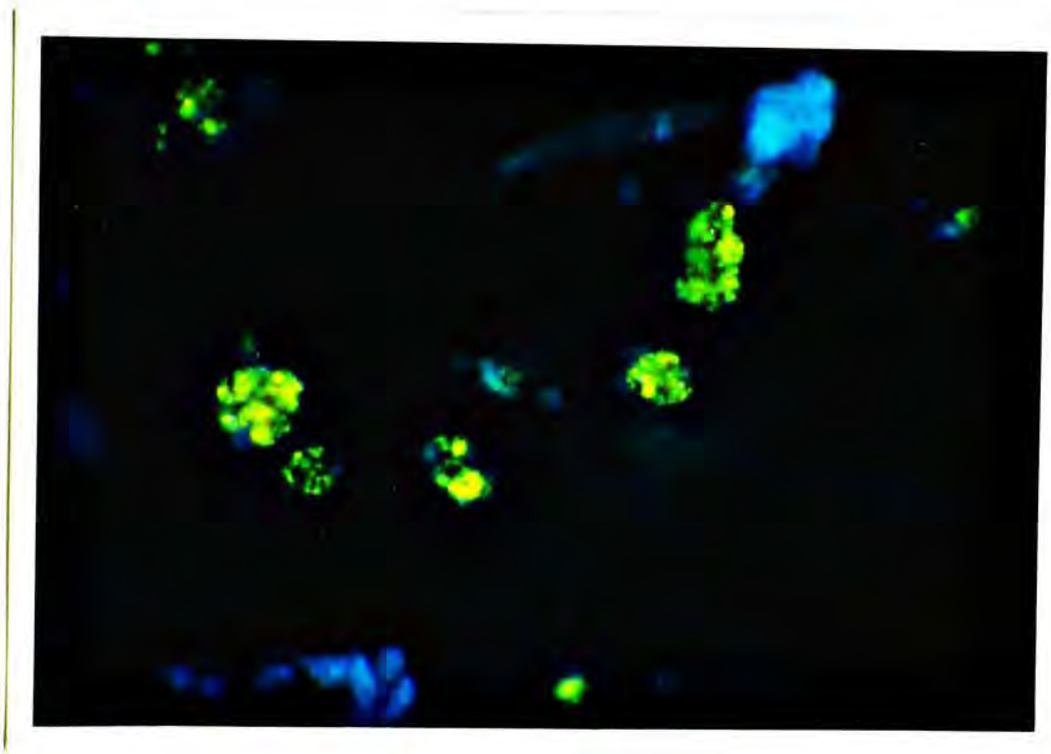

Fig. 4.14 Specimen from patient suffering from retinal detachment with previous cryotherapy showing bright autofluorescence of crowded lipofuscin-containing retinal pigment epithelium cells. (x 400)

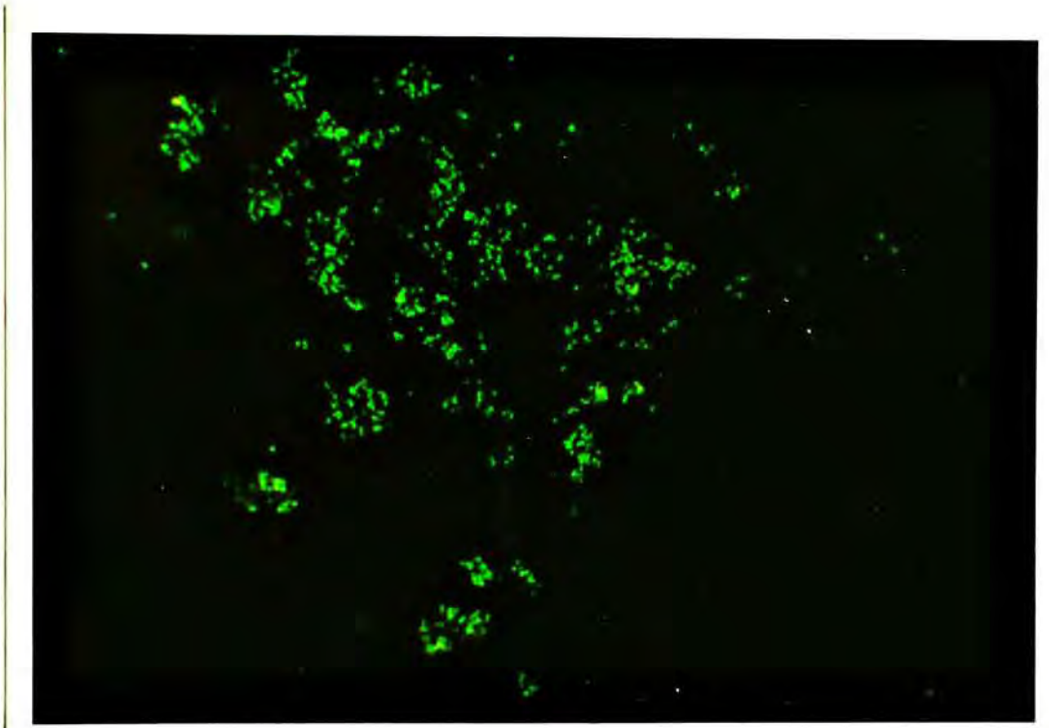

Fig. 4.15 Specimen from patient suffering from retinal detachment with previous cryotherapy showing a large number of autofluorescence of dispersive lipofuscin suggesting that many retinal pigment epithelium cells were disintegrated. (x 400)

CHAPTER 5

DISCUSSION

5.1 Evaluation of method for obtaining specimens

In the majority of previously reported studies, samples of SRF were usually collected at the time of choroidal perforation during external release of SRF. In view of the fact that SRF samples were collected externally, they were invariably bound to have some degree of contamination either from microscopic choroidal hemorrhage during perforation (27,29,88,89), or from tear and solution used during the operation.

To avoid these contaminations and to obtain as pure a specimen as possible, every sample in this study was collected internally during the initial stage of vitrectomy and aspirated directly from the subretinal space through the retinal break.

5.2 Total protein in subretinal fluid

Evidence obtained from experiments by others had suggested that ocular fluid dynamics in eyes with rhegmatogenous retinal detachment were severely altered. Aqueous humour flow was directed posteriorly into the vitreous humour, from which protein and fluid pass through the retinal break into the subretinal space. The water component was pumped from this latter space out to the circulation by the RPE, with sequestration of larger molecules within the subretinal space (90). In this study, the marked increase in concentrations of total protein in subretinal fluid as compared with vitreous humour supports this hypothesis.

The duration of the retinal detachment also affects the protein concentration of the SRF. Data obtained from this study showed that lower protein contents were detected in the subretinal fluid obtained from eyes with relatively short-standing detachment while long-standing detachments were associated with a higher subretinal fluid protein levels. This finding can in part be explained by the fluid dynamics of the subretinal space. Some investigators pointed out that normal unconcentrated vitreous contained so little protein as to leave scarcely any electro-phoretic trace. In contrast, subretinal fluid displayed a prominent elector-phonetic pattern (25).

Evidence, however, had also showed that in long-standing retinal detachment, the blood-retinal barrier could be broken down (91) and allowing the passage of higher-molecular weight proteins such as IgG and IgA into the subretinal space. This

might also lead to the elevated levels of total protein in subretinal fluid (28,29,38).

One previous study demonstrated that there was a continuous "infusion" of intravenously injected labelled plasma albumin into the subretinal space in detachment of all duration (92).

Evaluation of the total protein levels in subretinal fluids in different size of retinal breaks offered an indication of the degree of protein exchange between subretinal space and vitreous. With larger retinal breaks, the protein levels of subretinal fluids were relatively lower, indicative free diffusional exchange between the subretinal space and vitreous cavity. On the other hand, with smaller retinal break, higher protein levels were found. This suggests that the flow of fluid from the vitreous cavity into the subretinal space, and protein diffusion from subretinal fluid to the vitreous were especially hindered by small size of retinal breaks.

Akhmeteli and co-workers compared the biochemical findings with the clinical picture and showed that the levels of total protein in subretinal fluids were low in cases of retinal detachment which followed a relatively favorable course, whereas in severe cases, the levels were high (88).

In this study, however, the subretinal protein levels did not correlated with the severity of the retinal detachment. This may due to differences in duration of the

detachment, the level of vitreous protein, the integrity of the retinal pigment epithelium cells, intraocular fluid dynamics, and the size of the retinal break.

5.3 Basic FGF in subretinal fluids

Proliferative vitreoretinopathy is the main complication of rhegmatogenous retinal detachment which accounts for most failures in retinal reattachment surgery. This complication results from migration and proliferation of various types of cells, including cells derived from the pigment epithelium, and identifiable macrophages by the present study and other reports (20,93,94). The formation and contraction of preretinal membranes spreading along both surfaces of the detached retina and within vitreous lead to extensive retinal distortion and rigid vitreous strands. The cellular processes of proliferative vitreoretinopathy include cell activation, migration, proliferation and synthesis of extracellular matrix. In an experiment carried out by Vinores et al, it was suggested that DNA synthesis in all non-neuronal cell types within the detached region including the adjacent RPE cells was stimulated by retinal detachment (94). This also implies the possibility that detached retina and RPE could release substances into the subretinal space that stimulate RPE migration and proliferation. The stimuli that lead to their occurrence after retinal detachment remain to be determined. In this study, I have shown for the first time, that the level of basic fibroblast growth factor in the fluids bathing the retina correlated with the degree of PVR, using the method of an immunologic assay.

Analysis of bFGF levels in subretinal fluids in the present study revealed that as the severity of PVR increased, the levels of bFGF from these eyes likewise increased. On the basis of the above finding, it is possible that bFGF is somehow released into the subretinal fluids with the occurrence and development of proliferative vitreoretinopathy.

These findings also shed some light on the source of bFGF within eyes with PVR. During retinal detachment surgery, various forms of retinopexy are applied to both the choroid and retina in order to induce a localized scar to be formed in the region of the retinal break. It has been found that all forms of retinopexy including cryotherapy, photocoagulation and diatherapy cause significant breakdown of the blood-ocular barriers, thereby allowing serum components access to the intraocular space (95). It has been proposed that these serum components may play a role in inducing the proliferative vitreoretinopathy (95). In this study, however, bFGF was undetectable in all the patient serum samples using the same immunologic method employed. This suggests that there may be an intraocular source of bFGF. Because of very low levels of bFGF in normal human vitreous detected in this study, it was likely that bFGF was released from the retina after retinal detachment. Recent studies carried out by others confirmed this hypothesis. In 1992, bFGF was found both in the cytosolic and nuclear fractions of human retinal pigment epithelium (96). The bFGF had also been shown to be localized in the neuroretina to various cellular and extracellular location (11,12), including the interphotoreceptor matrix that

occupies the extracellular space between the neuron retina and RPE (13).

The underlying mechanisms by which bFGF do not normally cause a mitogenic response remain to be determined. A possible explanation for this is that bFGF exists normally in an inactive form which will only convert into the active form when needed (58). Another explanation for this is that the regulation of cell proliferation lies not with the concentration of a mitogenic growth factor, but rather with the cell itself. It is plausible that the cell regulates its receptors and then responds to the surrounding sea of growth factors when need arises(54).

By using linear regression analysis and F test of regression equation, it is not possible to establish a relationship between the bFGF content and the total protein concentration in subretinal fluids. In those subretinal fluid samples with high levels of protein, the levels of bFGF were not found to be uniformly increased. This may be due to the difference source of origin of these chemical parameters. The retina (including neuroretina and RPE) is the main source of bFGF in subretinal fluids, whereas, the protein in subretinal fluids could probably derive from blood or serum.

Basic FGF is a potent mitogen for mesoderm- or neuron-ectoderm-derived cells (9,52,97), triggering RPE cell proliferation and greatly reduces their average cellular doubling time. This is primarily due to a shortening of the G1 phase of the cell cycle (56). Although the mechanism by bFGF is poorly understood, this is the first report to show the presence of bFGF in subretinal fluids during the course of

PVR. The results from the present study suggested that severity of PVR was correlated with the level of bFGF in the subretinal fluids. It is possible that elevation in the levels of bFGF might be sufficient to stimulate migration and proliferation of vascular endothelial cells, fibroblasts, glial cells and retinal pigment epithelial cells. Basic FGF appears to play a role in the course of development of PVR.

5.4 Elevated level of bFGF in eye after cryotherapy

Higher levels of bFGF in subretinal fluids of patients with previous cryotherapy than those without previous cryotherapy support the conjecture that bFGF is probably released into subretinal fluid after cryotherapy from the damaged RPE and neuroretina. This study also showed that cryotherapy could release neuroretinal cells and destroyed RPE into subretinal fluid.

Since the elevated level of bFGF has been shown in this study to be correlated with the development of PVR and might be sufficient to stimulate migration and proliferation of RPE cell, it is possible that cryotherapy could be as one of the many exacerbating factors in the pathogenesis of PVR.

The role and presence of other growth factors or biochemical mediators in SRF deserve further investigation. However, because of the availability of the

limited amount of SRF samples, only bFGF was determined and analysed. Investigations carried out by others suggested that growth factors, such as acidic fibroblast growth factor, epidermal growth factor and insulin-like growth factor were also mitogens for retinal pigment epithelial cells (6,98). PVR appears to be the result of a very complex biological event.

5.5 Cell components in SRF of PVR

In this study, the cellular components of subretinal fluids surrounding the detached retina and proliferative fibrocellular membranes were also examined. The subretinal fluid accumulated in the space between the neuron retinal and pigment epithelial cells, which were frequently considered to be a major component of proliferative subretinal and epiretinal membranes. Using ultrastructural criteria, subretinal fluids were found to contain two cell types, pigment epithelial cells and macrophage-like pigment-containing cells (85).

Retinal pigment epithelium cells were found in all collected samples. These cells have long been regarded to be the main constituent of epiretinal membranes and the findings obtained in present study support this long standing observation.

The present study also suggest that ciliary or iris pigment epithelial cells could also contribute to this proliferative process.

By autofluorescent analysis, many pigmented cells are found to be totally devoid of lipofuscin and contained only melanin granules were found. Lipofuscin is thought to come from a deficient degradation of phagocytized in which it is shed in the outer segments within intracytoplasmic phagosomes (99) seen contained in the retinal pigment epithelial cells. Normal ocular histological sections clearly differentiate between lipofuscin-containing retinal pigment epithelium cells and ciliary or iris pigment epithelial cells that do not contain any autofluorescent granules of lipofuscin but only melanin pigment. The presence of these two distinct populations of pigmented cells, provides clues of the possible involvement of ciliary or iris pigment epithelial cells during the course of proliferative vitreoretinopathy.

Macrophages detected by immunocytological methods were also identified in some of the collected samples, but the number of cells was limited. The possible origin for these macrophages could derives from circulating blood. When a retinal wound was purposely created, it was unavoidable that there were blood components around the retinal break (21) and some white blood cells could enter the subretinal space. By using intravitreal injection of macrophages, experimental model of proliferative vitreoretinopathy had previously been induced (70).

Other studies, however, demonstrated that macrophages in subretinal fluids and proliferative tissues were transformed from retinal pigment epithelial cells (24,100) and remained negative for macrophage markers (24).

In many cases, there were a large number of neuroretinal cell in subretinal fluids specimens. It is possible that unsatisfactory anatomic and functional results which are observed in long term retinal detachments are not only caused by the presence of proliferative membranes in the subretinal space, but also by the destruction of a large number of neuropithelial cells.

Several studies conducted by others (73,74) that cryotherapy could cause release of RPE cells into subretinal fluid. It has been shown that serum components, including fibrin and fibronectin, help the migration of RPE cells into subretinal fluid and vitreous cavity (101,102). Cryotherapy causes breakdown of the blood-retinal barrier, allowing the release of serum components into the vitreous cavity. For these reasons, cryotherapy may well be one of the many causes in the pathogenesis of PVR (101).

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